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DINOFLAGELLATE TOXINS RESPONSIBLE FOR CIGUATERA FOOD POISONING

Annual Report

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Donald M. Miller

March 30, 1991

Supported by

US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-87-C-7002

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91-08595

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution authorized to U.S. Government Agencies only; Proprietary Information, December 20, 1989.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Southern Illinois University Department of Physiology 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Carbondale, Illinois 62901-6512	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-87-C-7002
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21702-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
DINOFLAGELLATE TOXINS RESPONSIBLE FOR CIGUATERA POISONING

12. PERSONAL AUTHOR(S)
Miller, Donald M.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 12/1/89 TO 3/31/91	1991 March 30	70

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	03		Ciguatera; Toxins; Inhibitor; NMR; $^1H^1H$ COSY
06	01		Dinoflagellates; Mass Culture; Purification, RAI

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

During the fourth contract year over 300 grams dry weight of the dinoflagellate Gambierdiscus toxicus was grown in large scale culture. Chemosystematic studies resulted in information which allowed improvements in both culturing and toxin production. Gram levels of cells and toxic extracts were processed in an effort to improve toxin production by G. toxicus. NMR characterization of suspected toxin was completed and is being analyzed. Crude and semi-purified toxins were delivered.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia M. Miller	22b TELEPHONE (Include Area Code) (301) 663-7325	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

SUMMARY

Ciguatera is a syndrome occurring in humans who have become intoxicated from eating poisonous fish. Fish sporadically accumulate the toxin through the food chain or directly from eating toxic dinoflagellates. Previous research points to the presence of multiple toxin involvement. Some of these toxins are purported to be ion channel inhibitors or activators. In addition to the establishment of facilities, this contract requires the growth of sufficient quantities of three different species of dinoflagellates to allow purification of milligram quantities of toxins for delivery to the U.S. Army Medical Research and Development Command. In this fourth year of the contract, growth of the dinoflagellate *Gambierdiscus toxicus* in mass culture has been successful beyond previous projections and expectations. Purification of the products of one of these toxins down to the 0.5 µg/MU level in milligrams quantities has been achieved. Further purification and interpretation of NMR data is still in progress.

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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STATEMENT OF THE PROBLEM

Ciguatera is one of several forms of food poisoning which occurs in humans resulting from the ingestion of toxic fish. The "ciguatera syndrome" is the result of toxins accumulated by fish through the food chain from dinoflagellates. Dinoflagellates produce a variety of toxins, some of which are ion channel inhibitors. In the case of ciguatera, the toxins can be accumulated through the food chain and stored by fish which are eventually consumed by humans. The ingestion of toxic fish produces a variety of digestive and neurological symptoms and sometimes death. At the present time, there is no adequate assay system for the detection and identification of the toxins. The structure and mode of action of these toxins is unknown. However, published results on crude toxins have described them as sodium and calcium channel inhibitors.[1,2] There is at present no known prophylactic or ameliorating treatment for ciguatera intoxication, notwithstanding a recent popular report of manitol therapy.[3]

The acquisition of milligram amounts of purified toxins would help to unravel the molecular structure of the toxins, their physiological actions and help to develop effective prophylactic treatment and effective countermeasures against the actions of the toxins. Our previous work on "ciguatera" resulted in the establishment of the SIU culture collection of toxic dinoflagellates. We proposed to grow three species of toxic dinoflagellates in mass culture for an extended period of time. After the cultures reached maximum growth they would be harvested and crude extracts made. The crude extracts would be assayed by mouse (LD_{50}) and an isolated bioassay preparation. Crude extracts would be purified by HPLC and liquid-liquid extraction. The purified toxins would be assayed the same as the crude and samples of purified toxin delivered to the US Army Medical Research Institute of Infectious Diseases.

BACKGROUND INFORMATION

Ciguatera Poisoning

Ciguatera poisoning is a syndrome which occurs following the ingestion of certain tropical marine reef-fishes that sporadically acquire toxicity. It is one of nine known forms of ichthyosarcotoxism: poisoning of humans resulting from eating fishes which contain poison within their musculature, viscera or skin.[4] Halstead[5] has implicated over 400 species of marine fishes as carriers, most, if not all, of which are an integral part of the food web of coral reefs associated with oceanic islands within a circum-global belt from 35°N to 34°S.[5,6]

The Ciguatera Syndrome

The symptoms that occur after eating toxic fish typically include both gastrointestinal and neurological manifestations. Typical symptomatology in humans has been reviewed and summarized by several authors.[7, 8, 9] Earliest symptoms of intoxication usually include gastro-intestinal upset, which may last for several hours or weeks. Moderate to severe intoxications usually produce neurological symptoms which may last weeks to months. Irregularities in nerve conduction parameters in fish,[10,11] mammals[12] and humans[13,14] have been documented. Thus, it is quite clear that intoxication affects the nervous system for extended periods of time. In at least one case of severe intoxication symptoms persisted for 25 years:[15] death may result and if so, it usually occurs within several days. In an isolated case, death has occurred within ten minutes and the fatality rate has been approximated as 12%[5] and 3%.[8] It is suspected that a large number of ciguatera intoxications, some from eating frozen fish, are not recognized as such.[16]

Multi-Toxin Involvement

A few early researchers suggested that the great variety of symptoms displayed by patients suffering from ciguatera and their inconsistent responses to certain clinical treatments indicated that there was more than one primary toxin causing the ciguatera syndrome,[17,18] while Banner et al.,[19] argued that ciguatoxin was the principal factor. Nevertheless, later studies coupled with the variability in results from testing of extracted fish tissues on a variety of preparations have emphasized the occurrence and importance of multiple toxins[20-25] Yasumoto et al.,[22] provided evidence that ciguatoxin was of exogenous origin and was not a metabolic product of primary consumers. These authors reported that an analysis of gut contents of *Ctenochaetus striatus* (a detrital feeder, exclusively) revealed a portion, designated as unidentified particles, containing a high concentration of "ciguatoxin". A recent report has indicated that ciguatoxin from both fish and dinoflagellate cells are modified forms of brevetoxin.[26] A second report has indicated that the toxin detected in ciguatoxigenic fish (eel) was palytoxin.[27] A most recent report has indicated that ciguatoxin both from cells and fish is a complex polyether structure.[28]

Dinoflagellate Toxins Affecting Ion Channels

The use of ion channel toxins has been crucial to uncovering the mechanisms of how ion channels work. It is well documented that two other toxins from dinoflagellates, saxitoxin and gonyautoxins are inhibitors of sodium channels. Brevetoxins have been found to affect sodium ion channels.[1,29] The current literature is somewhat confusing and indicates that ciguatera toxins act on sodium channels, potassium and or on calcium channels or all three by way of an intracellular messenger.

Dinoflagellate Involvement in Ciguatera

That dinoflagellates are the source of ciguatera-toxins has been well documented. In the Pacific, Yasumoto and others[24,30] obtained significant quantities of ciguatoxin from samples of detritus collected from dead coral near the Gambier Islands. The most toxic fraction of the detritus contained large numbers of a dinoflagellate, which he tentatively identified as "*Diplopsalis* sp." Subsequently, Adachi and Fukuyo[31] named the organism *Gambierdiscus toxicus*. Yasumoto et al.,[32] connected the production of toxin with both the dinoflagellate and toxic effects in mice.[32] The dinoflagellate, *G. toxicus* has subsequently been isolated from ciguatera prevalent areas near Japan,[32,33] and Hawaii.[34,35] McFarren and others[35] have provided accounts of ciguatera-like poisoning (*G. breve* ?) from shellfish collected from the west coast of Florida. Other investigators have published on *Gambierdiscus toxicus* from Florida.[36] Tindall and his group[37] have grown in mass culture and extracted toxins from three dinoflagellate species which were isolated from areas of the Caribbean in which ciguatera intoxication was prevalent. Thus far, three particular dinoflagellates are implicated in the production of ciguatera toxins: *G. toxicus*, *P. concavum*, and *P. rathymum*(= *P. mexicanum*).

Toxin Producing Dinoflagellate Strains

There is ample evidence to indicate that different strains of the same species of dinoflagellates produce different numbers and amounts of toxin.[38-40] There are also reports of loss of toxicity of dinoflagellates after culture. Under our conditions we have found that the initiation of mass cultures from unialgal or pure cultures has confirmed the strain differences but our cultures (*G. toxicus*) have produced toxins through continual subculturing since 1984. The same holds true for many of the other species.

Isolation of Dinoflagellate Toxins

Hashimoto[21] extracted toxic components from *G. toxicus* cells utilizing a boiling methanol extraction prior to doing a water-ether partitioning. The ether extracted portion was further treated with acetone to derive a toxic fraction. Since we have found this fraction to precipitate in very cold acetone we term it the ether soluble acetone precipitate (ESAP) fraction. Most cell isolation procedures used an initial partitioning of the cells with a water-ether mixture. The treatment of the water phase of the cell extracts have been similar by all investigators. After an acetone partitioning the filtrate is chromatographed to yield a water soluble toxic component.

The treatments for the ether phase of the cell extracts have differed. Yasumoto used a technique which involved an acetone extraction of the ether-water phase. Bagnis and others[41] modified the technique to include a cold acetone treatment that resulted in both ether-soluble acetone precipitates (ESAP) and ether-soluble acetone filtrates (ESAF). Even though he utilized this separation procedure, he then combined the ESAP with the WSAP fraction. Tindall and his group discovered that when the ESAF material was kept in the cold acetone all of the toxic activity eventually precipitated out of the filtrate. Thus, they adopted acetone precipitation as a step in the procedure hoping to further purify the toxic component. The toxic fractions obtained by these procedures differ with the particular dinoflagellate species.

Fish Toxins

The studies of toxins from fish extracts are difficult to draw conclusions from for several reasons. The most critical reason is that, if there are multiple toxins in fish (determined by their diet), and any one toxin may have different effects on a variety of assay systems, then it is possible that the extraction of toxin from the same species of fish by investigators from different locales will produce different symptoms.

From the foregoing we conclude, that if we want to determine if a particular fish is toxic, we must have a test(s) or assay system(s) which

is(are) specific at a known level for each of the particular toxins which may be involved in the ciguatera syndrome. For this express reason, we decided at the outset of our experimentation that the utilization of cell cultures would be the most productive approach.

Indeed, the same philosophy argued above for the diagnosis of toxic fish would apply to the treatment of the disease ciguatera. A different treatment would be called for were a person intoxicated with a sodium channel inhibitor rather than a calcium channel activator. Eventually, it would be ideal to have an assay system which would, in fact, reflect the number, kinds and amounts of toxins. The construction of these specific chemical tests is only possible, however, if one has reasonably pure toxin, which is separated from other toxins and identified.

These constraints have dictated our approach to the entire problem since we started our research in 1978, as follows:

1. Identify sources of the toxins.
2. Produce large amounts of toxic organisms.
3. Improve extraction techniques.
4. Find a sensitive bioassay(s) for screening.
5. Use bioassay to assist in purification.
6. Improve purification techniques.
7. Use purified toxins to:
   a. investigate physiology.
   b. investigate structure.
   c. elaborate chemical assay system.

Our preliminary works and the work of others[37,42-44] have identified several different toxins which may be involved in the ciguatera syndrome:

1. Ciguatoxin
2. Maitotoxin
3. Slow acting toxin (unidentified)
4. Scaritoxin-like toxin
5. Okadiac acid
6. Fast-acting toxin I (unidentified)
7. Fast-acting toxin II (unidentified)

Clearly, the understanding of the entire problem of the toxins produced by dinoflagellates requires a definitive test or set of tests which will allow us to discriminate between the toxins and be able to quantify and follow them. Other desirable features of a test procedure would be 1) provide the same baseline for each of the toxins, 2) consume only a small amount of toxin, 3) require a small amount of time for the assay, and 4) be able to detect modifiers of sodium channels in membrane as well as calcium channels.

Significance

"Ciguatera-toxins" are involved in a variety of short term symptoms for which people commonly present themselves to a doctor for treatment (e.g. diarrhea, headache, etc). In addition intoxication can result in prolonged disability or even death. Another aspect of the toxins and one which has yet to be addressed is the long term effects on animals that is: are they cumulative, are there storage sites, what are the affected sites, and how long before excretion? The reoccurrence of neurological symptoms years after intoxication would seem to indicate a retention of the toxin and/or toxic effects in the nervous system.

The toxins also include a novel calcium channel inhibitor[2] and a sodium channel inhibitor.[1] In addition, there is the prospect of discovering other new and important ion channel inhibitors. Ion channel inhibitors have been essential to our present understanding of ion channel physiology and structure. Clearly, the identification, isolation and purification of individual toxins involving ion channels will expedite (1) an understanding of their structure, (2) allow the investigation of their physiological actions, (3) expedite the formulation of ameliorative and prophylactic treatments, and (4) allow the elaboration of a specific chemical assay.

APPROACH TO THE FOURTH YEAR OF THE STUDY

Methods for Growth of Cells

The dinoflagellate cultures which we used for this project are part of the Southern Illinois University Culture Collection, housed in the Department of Botany. At present this collection houses strains representing dinoflagellate species isolated from "ciguatera community" areas of the world. Our stock cultures are routinely grown in 50 ml volumes in 125 ml Erlenmeyer flasks. The medium is ES Medium[45] made with natural seawater, with 1.5% soil extract added. These cultures are kept in refrigerator-type culture chambers at 27°C and 500 ft-c. cool white fluorescence illumination (either continuous or on a 16:8 light-dark cycle, depending upon the requirements of the particular species). Stock cultures are transferred every 7-10 days. Two generations of cultures are retained as back-ups to the new transfers. The subculturing and maintenance of triplicate cultures is labor intensive and requires approximately 20 manhours per week by an experienced person. In addition, all cultures are examined periodically by one of us to check for contamination. Currently we maintain stock cultures of over 153 strains of dinoflagellates. Preparation of the growth medium requires millipore filtration (0.45 or 0.22 μm) and sterilization of the sea water, sterilization of the flasks, compounding of the growth medium, innoculation and siting in the growth chambers. Conservatively, this requires approximately 20 manhours per week for a total of 18 carboys. Because the growth cycle for both the subcultures and the mass cultures takes four weeks, we initiate a mass culture every two weeks. It is critical for the cultures, to achieve the maximum toxin production, that they be harvested very close to the 30 day period.

The development of mass cultures involves transferring cells from stock cultures to a series of two liter fernbach flasks containing enriched seawater medium. After the early stationary phase of growth has been reached (approximately 15-20 days) each of these cultures are used to inoculate 18 liters of the same medium in 20 liter carboys. Mass cultures are grown under the same light and temperature regime

as noted above and are aerated continuously in order to prevent CO_2 depletion and to provide moderate agitation. Cells from small cultures are harvested by centrifugation or filtration. Cells from mass cultures are harvested by means of a Pelicon concentrator using 0.45 μm membranes after cultures reach the early stationary phase of growth (30-35 days). If the culture has excessive amounts of slime it is first sieved before the use of the Pelicon.

Chemosystematic Studies

Systematics is a branch of taxonomy which deals with assessing variation in characters between and within genera from living material.[46] A systematic study has been incorporated into this project as a means of identifying clones (cultures initiated from individual cells) which are inherently good producers of toxins. Our culture collection now includes more than fifty clones of *Gambierdiscus toxicus* from a variety of locations including Bermuda, the Bahamas, Florida, the Caribbean, Hawaii, Australia, French Polynesia, Mexico, Fiji and other areas. It is this diverse, bank of living material which forms the basis for the systematic studies.

Our first approach in assessing clonal differences was to acclimate the *G. toxicus* clones to the same conditions (light, temperature and medium) in one liter cultures. Once acclimated, the final one liter culture were harvested and also used to inoculate an additional one liter culture which served as the starter for a 15 liter culture. The toxicities of the crude methanol cell extract from the acclimated one liter cultures were compared in terms of the number of mouse units per milligram of dried cells. This is a quantitative measure of clonal toxicity, and does not consider qualitative differences in toxins. We assert that because of the acclimation process the varying potency of these extracts among clones is due to interclonal genetic differences and not to environmental differences.

Beginning in 1989 the extract potencies of several clones from the first and the last one liter culture were compared in order to assess the significance of the acclimation process. In addition, the potency of extracts from clone 135 were compared monthly in order to determine

when acclimation was achieved and once achieved, how stable a character it is. An additional control experiment was run with the Martinique (Caribbean) clone (175).

Clone 175 has been sub-cloned so that we can assess any toxicity differences which may result from micro-environmental differences in the culture chambers. Only toxicities which exceed the methodological errors were used for "chemosystematic" comparisons ("chemo" referring to the toxins and "systematic" to the clonal comparisons). The material from the 15 liter cultures was used to assess qualitative differences in toxins among the clones.

The genetic comparisons indicated that clone 175 produces more toxin per unit weight than approximately twenty other clones of *Gambierdiscus toxicus* surveyed. Consequently, this clone was selected for physiological experiments designed to enhance toxin production, hopefully including the lipid-soluble toxin. The first phase of this work involves examining the macromolecular components and toxin production of clone 175 when grown under seven different light intensities and at three temperatures. These are 378, 648, 1081, 1999, 3350, 3782 and 4300 lux light intensities and 28°C, 25°C and 22°C temperatures.

The cultures were acclimated to each temperature following the methods of Brand et al.[47] and Bomber et al.[48] All cultures are grown in a Percival culture chamber equipped with microprocessor controls. The microprocessor enables us to change temperatures slowly using a constant gradient. The cultures were harvested through a 32 µm screen and lyophilized for approximately 24 hours until dry.

Kochert[49] determined protein from cell pellets that were first extracted with chloroform/methanol. Total lipids were then determined from the organic solvent extracts. We determined that as much as 10% of the protein will enter the organic solvents. Thus, we analyzed proteins and lipids separately. The dried cells were extracted with 1N NaOH with sonication followed by immediately adjusting the pH to approximately 7.5 with distilled, deionized, charcoal filtered water. This method yielded far better protein extraction than SDS (sodium dodecyl

sulphate) or the method of boiling the cells in 1N NaOH[49]. The extract was filtered through a 0.2 µm screen and analyzed for proteins by the Bio-Rad micro-method. Carbohydrates contents were determined from the same extracts via the sulphuric acid method.[49,50] Lipids were determined from separate samples following Freeman et al.[51] and Sperry and Brand.[52]

The chlorophyll content was determined from the equations of Jeffrey et al.[53] Carotenoids were determined via the methods of Jensen.[54] All samples were processed in darkened fume hoods and stored under nitrogen for best preservation. Most samples were analyzed within two weeks of harvest. The potencies were determined as before (previous quarterlies) using the linear interpolation tables of Weil.[55] Ammonium and phosphate uptake rates were determined from disappearance of the nutrients from the culture medium following the methods of Strickland and Parsons.[56]

Previous work[57] has determined that it is not possible to completely identify a systematic "variant clone" or "race" of *G. toxicus* by analyzing only one character, e.g. extract potency. Consequently, this project includes an "acclimated reproduction rate comparison".[47] In this study the response to light of different intensities by the different clones of *G. toxicus* is being monitored. Four light intensities are being used and include 80, 160, 205 and 250 footcandles of illumination. The reproduction rate (divisions per day) of the clones is plotted against the light intensity and the resulting slope of the line is used as a numerical systematic character. It is critical that the cultures be completely acclimated in this study, as in the toxicity study. The slope of the line is a valid genetic character because the reproduction rate is under enzymatic control and varies with the efficiency of enzymatic transcription (hence chromosomal differences) under different environmental conditions. These data from these studies will also be useful as a data base in physiological studies which will examine the effect of light on toxin production.

Seventeen clones of the ciguatera-causing dinoflagellate *Gambierdiscus toxicus* were physiologically adapted to the same

environment over several months. There were significant variance components detected between non-acclimated and acclimated cells for the cell potencies, yields and reproduction rates of these cultures.

Toxin Potencies

Outbred Harlan Sprague Dawly ICR(BR) mice weighing approximately 20 g were used to assess quantitative differences in the potencies of the methanol extracts. The potencies were then determined as LD_{50}'s for each clone using from 3 to 10 mice at each of 4 dosage levels. The mice were observed for 48 h. The LD_{50}'s were determined from the linear interpolation tables of Weil.[55] Alternatively, in some cases the LD_{50} was determined by linear regression.[58] The final values are expressed as the number of mouse units (LD_{50} dose for a 20 g mouse) per mg of dried cells, per cell of *G. toxicus* and in terms of number of cells per mouse unit.

The acclimated and non-acclimated cell potencies were compared in the same manner as the growth rate and cell yield statistics. In addition, a one-way Analysis of Variance with Replication[58] was used to compare selected clones from broad areas. The f_{max} test[58] was used to assess homogeneity before conducting all ANOVA's The potencies used in this ANOVA were plotted vs. latitude of collection along with the values for all other clones. The clones used in generating the ANOVA are also plotted with their 95% comparison intervals, calculated from and a-posteriori T-method comparison among means.[58] Potency and latitude were also tested for correlation by the Pearson test.[58]

Three clones (177, 350 and 135) were also assayed for potency at 6 to 11 points during the growth cycle and these were plotted vs. days in culture and compared. The two parameters were then tested for correlation by the Pearson test.[58] For clone 177, 3 of 11 samples were assayed for potency 4x and compared by one-way ANOVA to test for significance of variation in potency through the acclimation process.

The means from this test were also compared by the T-method. The potency and reproduction rate of clone 177 was also monitored after continuous batch culturing was stopped and transfers were made in stationary phase. Potency changes for clone 350 were monitored through acclimation to the vita lite bulbs and then when returned to a cool white light environment at a similar light intensity.

As a control experiment on sub-clone variability, clone 175 was sub-cloned 25x, all isolates survived and 4 were selected randomly to determine the coefficient of variation among fully acclimated sub-clones. As a control experiment on the stability of the acclimated condition, clone 135 is still being monitored for potency data accumulation beyond one year.

Five other relationships were also explored by correlation analysis and include potency vs. cell size and potency vs. reproduction rate for all clones examined. The former test was performed on acclimated potencies only whereas the latter test included all data points. Cell size was determined as the transdiameter and computed from a minimum of 20 cells of each clone collected from a log-phase culture. The potency was also tested for correlation with chlorophyll a, chlorophyll c_2 and peridinin content (pg $cell^{-1}$). Pigments were assayed via the methods of Jeffrey et al.[53] and Indelicato and Watson.[59] They were subsequently extracted and analyzed for composition of proteins (Bio-Rad method), lipids[51,52] and carbohydrates.[49,50]

The initial methods for the extraction of the toxins is very similar to what is currently being utilized by other investigators (especially those attempting to isolate toxin from fish tissues) so that, to some extent, we can compare the toxic fractions which we obtain with what is in the literature. The cells are no longer extracted by refluxing in boiling aqueous methanol. Instead, extraction is now accomplished by crushing of cell, followed by sonication in methanol at room temperature. The methanol extracts are concentrated and subjected to liquid-liquid partitioning followed by cold acetone precipitation of the toxic components.

Further purification of the toxin will be either by thin layer chromatography (TLC), silicic acid chromatography or high pressure liquid chromatography (HPLC). Eluting solvents for column chromatography consist of chloroform-methanol (1:1), and chloroform-methanol-water mixtures (9:1:1). The eluting solvent for HPLC is methanol (100%).

Purification of Toxins from Mass Culture

Separation is achieved using three HPLC instruments associated with a single computer controller system. All three are Waters Company instrumentation and consists of a Delta 3000 Preparative-Semipreparative HPLC and a Model 300 Analytical system all interlinked with SIM modules to an 840 Controller System. While we use C-18 or C-8 columns in each system, the sizes differ, having 15μ in the Preparative and Semipreparative and 10μ or 5μ in the Analytical.

Mouse Bioassay

The mouse bioassay is the officially recognized toxicity assay for ciguatera recommended by the Official Organization of Analytical Chemists and the FDA. In addition, it provides a base line against which we can compare our isolated preparation assays. The carrier for toxic extracts is normal saline containing 0.5 ml of a 1% Tween-60 solution. Toxicity is determined by an intraperitoneal injection of 0.5 ml of a suspension of extract into approximately 20 g mice (Strain CRE:CD:BR:ICR). Toxicity is defined as death of the mouse within 48 hours. LD_{50} values are calculated according to the method of Weil.[55] Four dosage levels are used with three repetitions at each level. The LD_{50} is calculated from moving average interpolation tables.

Ileum Assay Procedures

We have utilized the terminal portion of the guinea pig ileum to assay dinoflagellate toxins. A description for the experimental setup for the ileum assay has been published.[60]

Guinea Pig Ileum. Female guinea pigs (350-600 g) were sacrificed by a cervical dislocation. A 2-4 cm segment of the terminal ileum was

removed and placed in physiological saline solution (PSS) at 37°C. The terminal portion will, however, respond to exogenously applied agonists. Hence its suitability for use in an assay system.

Tissue Bath. The excised terminal ileum is allowed to come to temperature in saline for 15 min then a one cm segment is cut and inserted in an Anderson type tissue chamber[61] modified as suggested by Bartelstone.

Water Circulating Bath. The guinea pig ileum is extremely temperature sensitive and changes as small as 0.3°C may affect its contractility and therefore your results. For this reason you must have a circulating water bath or some other means of controlling the temperature. The circulating water bath that we presently use is Fisher Model M8000, Isotemp, Constant Temperature Circulator. The ileum is connected to a locally-made device which is a true, isotonic-tension transducer. The counter weight we use is 2 grams. Amplification of the transducer signal is achieved by the use of a DC oscilloscope with a pen output. For storage of records and recording of data for calculations we chose an inexpensive chart recorder (Fisher Recordall Model 5000). Any good DC level recorder can be used, but a ten inch recorder makes the task of measurement much easier. The ileum has been shown to respond to acetylcholine, histamine, substance P, epinephrine, and several other compounds. Indeed, it has varying sensitivity to each of these compounds. We have found that the toxic fractions will give different inhibitions with different agonists.

Protocol for Reversible Toxins. The first protocol followed is utilized when there is only a reversible toxin in the extract. Initially a control series of acetylcholine or histamine stimulations is performed at different dosages to determine three doses which would give us values between 20 and 80% of maximum contraction of the ileum segment. That particular set of test doses is then utilized throughout the rest of the experiment. All subsequent responses of the ileum to agonist challenge are expressed as a percent of control values.

The toxin containing physiological saline solution is utilized for subsequent washes. Thus when testing for reversible inhibition, the toxin is always present in the PSS at the same concentration.

Non-reversible Assay. The second protocol is utilized when it is suspected that there only a non-reversible toxin in the extract. First a control series of histamine stimulations is performed at different dosages to determine a dose which would give us approximately 80% of maximum contraction of the ileum segment. That particular test dose is then utilized throughout the rest of the experiment. All subsequent responses to histamine are expressed as a percent of that control value.

Tests for Purity of Toxins

The easiest manner to test for purity of the toxin is to run in a recycle mode on the analytical HPLC to determine if after a given number of recycles the single peak remains or resolves into more than one peak. We will use several criteria for testing for purity: first the presence of single peak after recycling on analytical HPLC; second, migration on TLC plates in different solvent systems, and structural data from Nuclear Magnetic Resonance.

NMR Spectroscopy

At the present time Nuclear Magnetic Resonance (NMR) would provide us with the most informative data and yet is non-destructive to the sample analyzed. For these reasons it is the method of choice. Samples of purified toxins will be sealed in special, thin-walled, small-bore, NMR sample tubes from Wilmad Glass Co. Two state of the art NMR instruments are presently available for our use: specifically a 300 MHz Varian VXR-300 and a 500 MHz Varian VXR-500 multinuclear spectrometer system. Both instruments operate in the pulse Fourier Transform mode and are equipped with a liquid helium VXR superconducting magnet and acquisition hardware. Both have H_2 fixed frequency lock system with 5 mm broad band computer switchable probe. Multiple probes to two chambers accepting, 5 mm (narrow bore)

and 10 mm (medium-wide bore) tubes for ^{1}H, ^{13}C, ^{31}P, ^{15}N, ^{19}F and other nuclei. Quadrature detection. Homo- and hetero-nuclear decoupling with spectral limits of 100 to 100,000 Hz. Variable temperature control (-70° to 140°) under computer control for all probes. VXR data station with dual high density disks. The VXR-300 operates at 300 MHz ^{1}H resonance and is presently used primarily for ^{13}C analysis. Automatic performance of standard relaxation experiments, as well as data recording is accomplished by an associated computer. The chemical shifts recorded are then interpreted for structure.

Packaging of Toxins for Delivery

Purified toxins in 100% methanol will be placed into vials, concentrated to almost dryness under nitrogen gas, sealed and labeled for shipment. The vial is then encased in a plastic container with absorbant material. The plastic container is then packed into a metal container and sealed. This is enclosed in packing material and styrafoam box surrounded by a cardboard container. The entire package is shipped by overnight express. Telephone calls are made before shipment to notify the commanders representative of the shipment and follow up calls are made to ensure delivery.

RESULTS

Culturing

In September of 1988, growth of clone 350 was suspended and all mass culturing was directed towards clone 175, because of the greater toxicity of this clone. Our previous success in culture maintenance and mass culture has continued during this year. At the end of 1989 we acquired the French Polynesian and several Australian clones and have been cultivating them through the acclimation process with a great deal of success. At the end of this year (1990) we acquired over 50 Australian and Fijian clones. These are now in the acclimation process. Starting in November of 1990, we reduced the growing of clone 175 in mass culture. At the same time we started mass culturing clone 350, French Polynesian (clone FP100) and Australian clones.

Acclimation and Comparison of Potencies of New Clones of *Gambierdiscus toxicus* from Australia and Mexico.

A series of large-scale acclimations including 5 clones from Australia (GT-A9, A11, A12, A14, A16), 1 clone from the Virgin Islands (GT-350) and 1 clone from the French Polynesian Islands (GT-FP100) was initiated in June 1990. Three clones from Cosumel, Mexico (GT-CM, CM1, CM2), 1 clone from Martinique (GT-175), 1 clone from Burmuda (GT-135), and an additional clone from Australia (GT-AA1) were added to the acclimation series in September 1990. Results of production in this series through September 10 are shown in Table 1. Growth rates will be determined for all cultures in this series. Once acclimated growth rates are achieved, the series will be terminated and potency of methanol extracts from each culture will be determined. Results from these experiments will be compared with results on 17 clones of *G. toxicus* presented by Bomber, Tindall, and Miller (1989). Also methanol extracts will be subjected to our standard purification procedures. Results on types and potencies of toxins from the various clones will be compared with our previous findings and those reported by other laboratories.

Table 1
Volume of Media and Cell Production in Continuous Acclimation Cultures of 7 Clones of *Gambierdiscus toxicus*.

Clone	Dates of Initiation - Harvest	6/20-7/17	7/17-8/4	8/4-8/21	8/21-9/10
GT-A9	Vol (L)	41.00	8.20	19.30	19.05
	Cell Mass (mg)	1908	563	1027	1211
	mg/L	46.5	62.2	53.21	63.57
GT-A11	Vol (L)	10.00	7.25	19.50	19.00
	Cell Mass (mg)	395	317	981	1095
	mg/L	39.5	40.28	50.31	57.63
GT-A12	Vol (L)	19.5	8.00	19.6	18.10
	Cell Mass (mg)	834	309	638	852
	mg/L	42.8	35.25	35.41	47.07
GT-A14	Vol (L)	40.50	8.00	20.10	19.55
	Cell Mass (mg)	1604	436	910	1078
	mg/L	39.6	49.25	48.06	55.14
GT-A16	Vol (L)	19.70	7.75	19.80	18.65
	Cell Mass (mg)	561	232	607	846
	mg/L	28.5	27.48	30.66	45.36
GT-350	Vol (L)	10.00	7.50	18.60	18.85
	Cell Mass (mg)	306	251	603	559
	mg/L	31.0	31.2	33.27	29.66
GT-FP100	Vol (L)	19.50	7.50	19.5	19.60
	Cell Mass (mg)	560	215	641	696
	mg/L	28.7	26.13	34.97	35.51
TOTALS	Vol (L)	160.2	54.2	136.4	132.8
	Cell Mass (mg)	6186	2323	5407	6337
	mg/L				

Physiology and Potency of the Toxic Dinoflagellate, *Prorocentrum concavum* During One Complete Growth Cycle.

Previous studies on growth physiology and acclimation of *P. concavum* (SIU clone 364) have been reported (Aikman and Tindall 1989). This clone was cultured in h/2 medium at 25°C under five light regimes (312 lux to 2690 lux). Determination of biochemical differences were made on each culture in a series leading to acclimated growth

rates under each light regime. Growth rates of *P. concavum* during the acclimation process increased initially and eventually leveled off to a constant rate (i.e. 0.36 div/day after 4-5 transfers at 2690 lux). Growth rates diminished with decreasing light intensities (0.36 at 2690 lux to 0.04 at 312 lux). During the two month acclimation period (at 2690 lux) total protein increased from 2930 pg/cell to 4130 pg/cell and total carbohydrates increased from 745 pg/cell to 1080 pg/cell. Pigment compositions of acclimated cultures over the five light regimes expressed as a carotenoid:chlorophyll ratio, exhibited a bell-shaped curve. The data indicated that both light intensity and acclimation have a profound affect upon the growth and physiology of *P. concavum*.

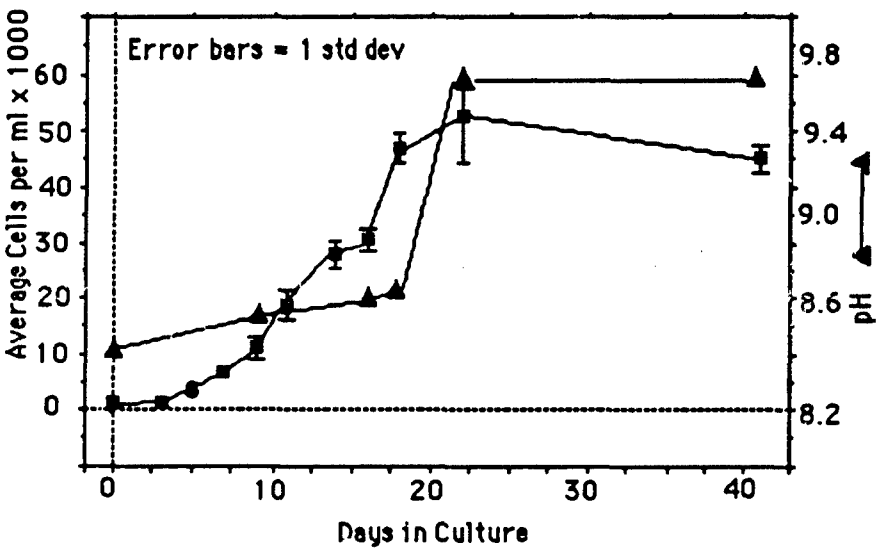


Figure 1. Cell densities and pH measurements of the complete growth cycle of *Prorocentrum concavum* (SIU clone 882a) grown in large-scale batch cultures (16 x 10L) containing h/2 enriched natural seawater medium under 16:8 light/dark cycle (1800 lux) at 28°C.

The present study was initiated in order to achieve the following objectives: (1) to determine changes in physiology of cells during one complete growth cycle; (2) to compare synthesis of FAT and OA with that of other biochemical constituents; (3) to enhance our ability to manipulate production of toxins; and (4) to determine the significance of *P. concavum* as a possible contributor of toxins to the ciguatera syndrome in humans.

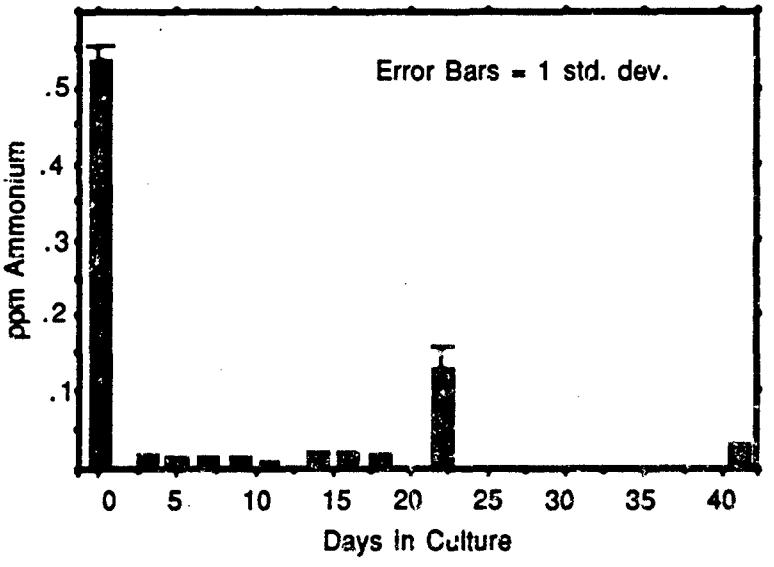


Figure 2. Depletion of Ammonium from the culture medium (h/2) over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

Preliminary Results and Discussion. The cell densities obtained from triplicate counts over the entire growth cycle of *P. concavum* ranged from 500 to 55,000 cells/mL (Fig. 1). Large-scale cultures displayed a sigmoid growth curve with a short lag-phase, followed by an active log-phase (0.35 div/day) and stationary phase. Concurrent measurements of pH in the culture media showed a significant increase at the end of log-phase (pH 9.6) which was not controllable with bubbling of sterile air (Fig. 1).

Systematic measurements of nutrient depletion in the culture medium revealed that the levels of nitrogen (NH_4) and phosphate in the culture medium were significantly reduced by early log-phase (Figs 2 & 3). However, nutrients were not deplete or limiting, since logarithmic growth continued long after measurable amounts of these nutrients had been extracted from the growth medium.

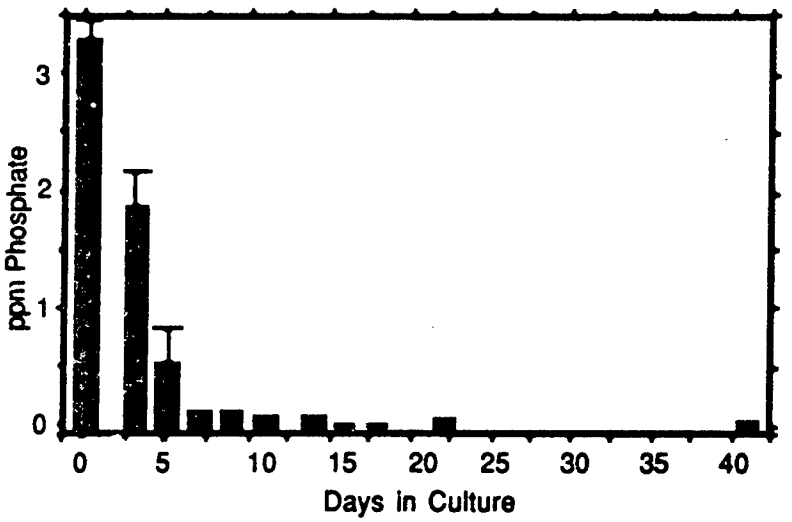


Figure 3. Depletion of phosphate from the culture medium (h/2) over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

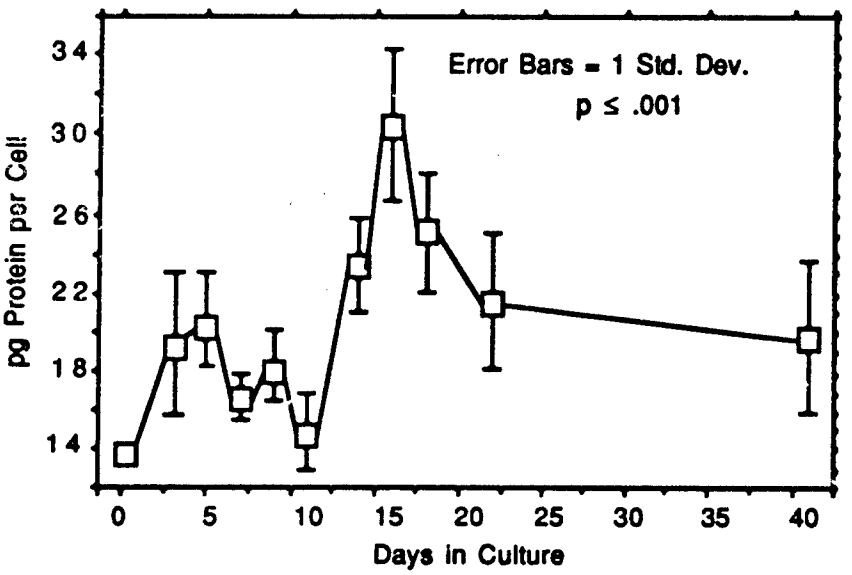


Figure 4. Cellular content of protein over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

Several biochemical parameters were measured to assess the physiological condition of the cells over this complete growth cycle. These parameters included total soluble proteins, lipids, reducible sugars (carbohydrates), pigments and toxicity. Figure 4-6 shows the relationships between protein, lipid and carbohydrate content during

culture development. Protein content increased initially from 1400 pg/cell to 2000 pg/cell but returned to the initial level around 1400 pg/cell after 12 days of growth. This suggests that during the log-phase, proteins are subjected to rapid turn-over rates.

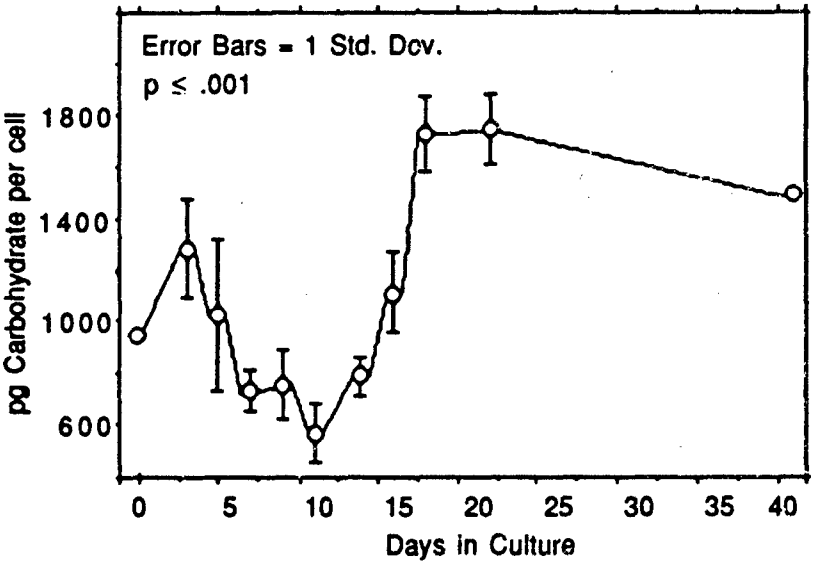


Figure 5. Cellular content of carbohydrate over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

As the protein level increases during the mid-log phase to around 3100 pg/cell (possibly due to slower turnover rates), total carbohydrates and lipids follow closely behind, reaching their maximum levels of 1800 and 1900 pg/cell respectively near late-log to early stationary phase.

However, protein content then decreases to early log-phase levels (2200 pg/cell) at the onset of stationary phase, indicating their degradation to near basal levels.

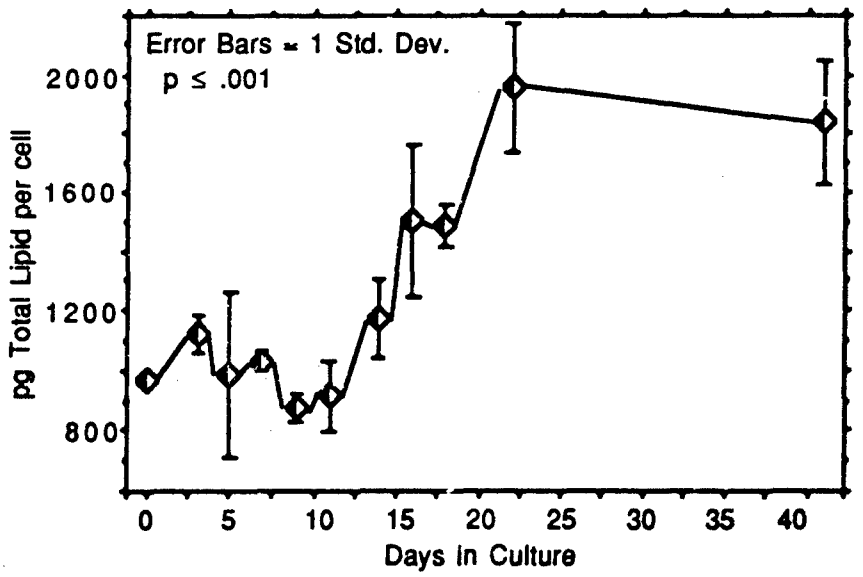


Figure 6. Cellular content of lipid over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

The cellular content of chlorophyll–a and peridinin follow the same developmental pattern as protein, with maximal cell content of 65 and 40 pg/cell respectively during mid-log phase followed by a transient drop in early stationary-phase (Fig. 7 & 8). Nevertheless, chlorophyll c2 content was maintained at mid log-phase levels until late stationary-phase. This suggests that either the metabolic machinery for the production of chlorophyll c2 is functional for a longer time period or the degradation of this pigment is slow. The indestructible nature of chlorophyll c2 is also observed in the laboratory. It is relatively insoluble in most organic solvents, it is difficult to separate from toxic fractions of *G. toxicus*, and it persists in extracts long after the degradation of chlorophyll a.

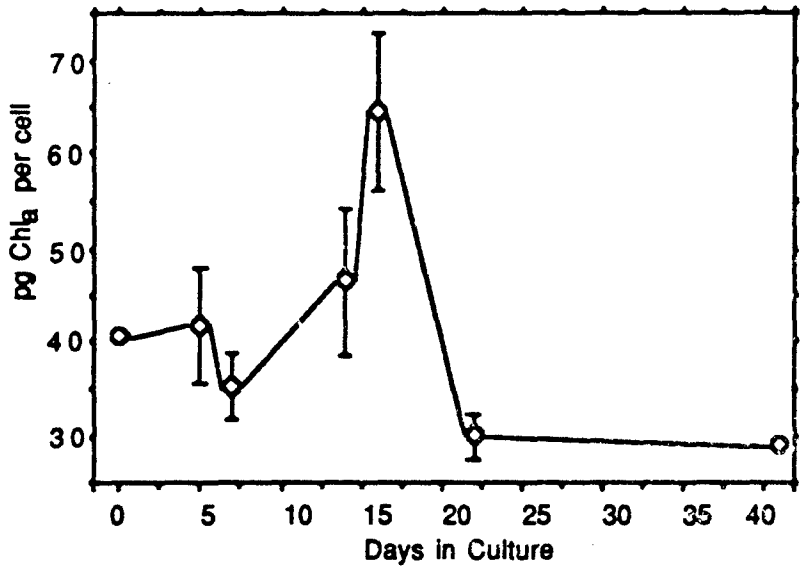


Figure 7. Cellular content of the pigment chlorophyll-a over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

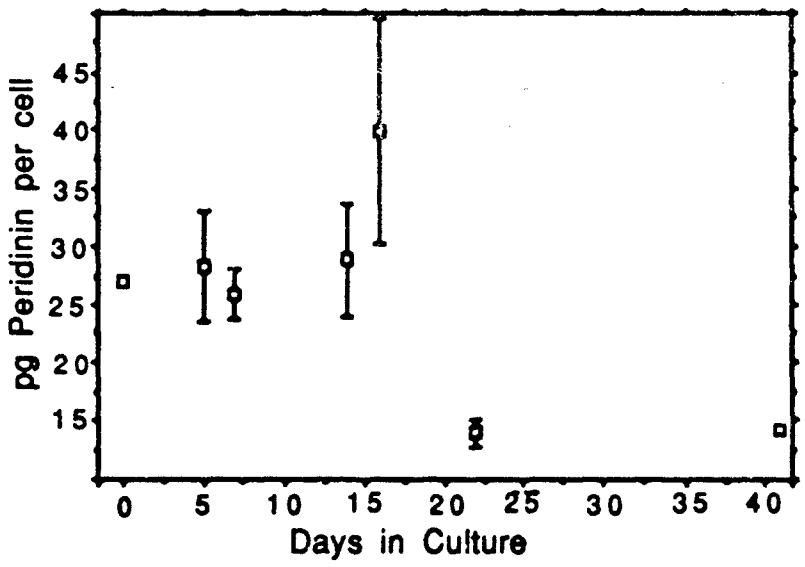


Figure 8. Cellular content of the pigment peridinin over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

However, this persistence may explain the more or less basal levels of protein maintained in the latter phases of growth, indicating a possible role of chlorophyll c_2 in the survival or protection of *P.*

concavum cells under unfavorable conditions (i.e. low nutrients, high pH, high light and shading).

Measurements of the potency of the FAT were accomplished using the mouse bioassay. Okadaic acid (OA) was determined using an enzyme-linked immunosorbent assay (ELISA; UBE Industries, Japan). Although these toxins appear to be chemically heterogeneous (FAT is water-soluble and causes similar antagonistic effects on the guinea pig ileum much like maitotoxin from *G. toxicus*; whereas OA is lipid-soluble and causes gastrointestinal distress and appears to inhibit specific phosphatase enzymes), the pattern of *de novo* synthesis was strikingly similar (Figs 10-13).

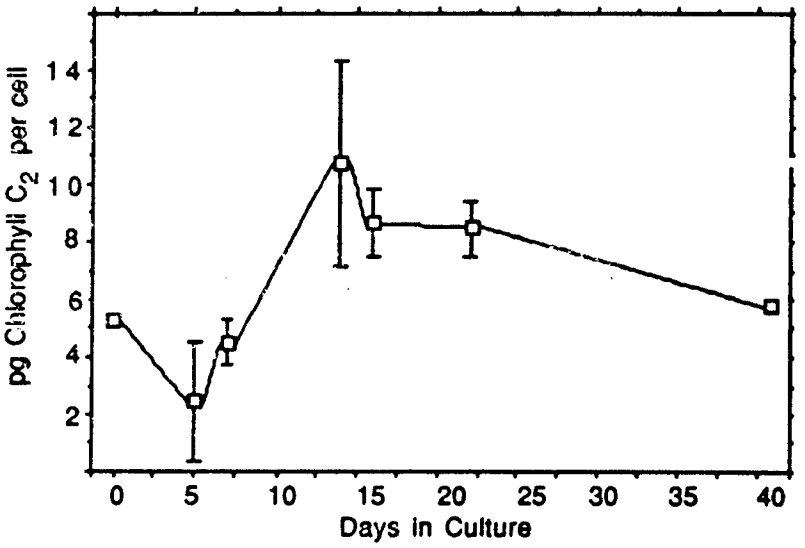


Figure 9. Cellular content of the pigment chlorophyll-c2 over the growth cycle of *Prorocentrum concavum* (SIU clone 882a).

Interestingly, the plots of toxin content were consistent with plots of biochemical constituents, although the highest correlation existed between toxin content and lipid content per cell (r = 0.975 for FAT vs lipid). This relationship is especially interesting since both compounds may have common acetate precursors (Shimizu *et al.*, 1990).

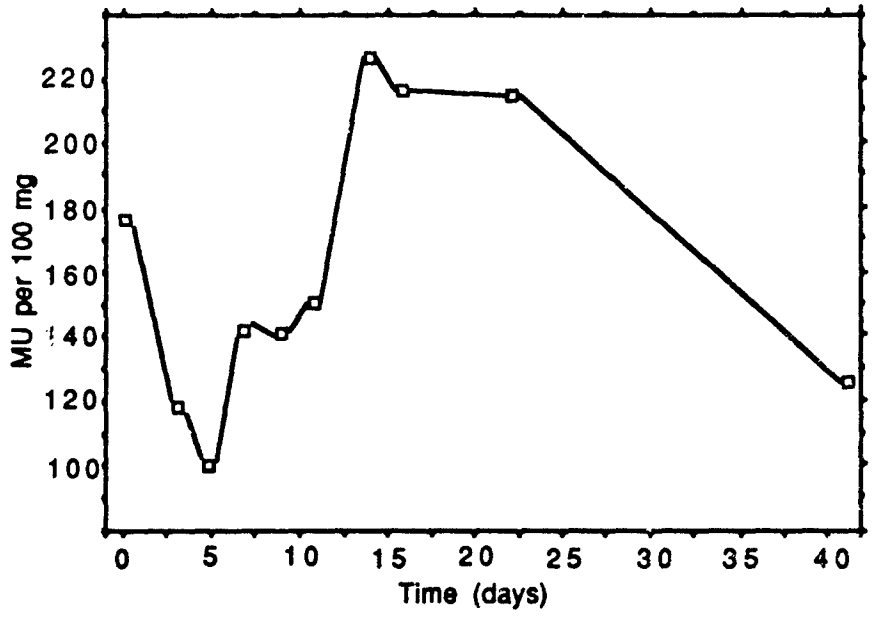


Figure 10. Cell potencies of Fast Acting Toxin as determined by mouse bioassay. Total extractable mouse units (1MU=LD50 dose) per 100 mg dry cells.

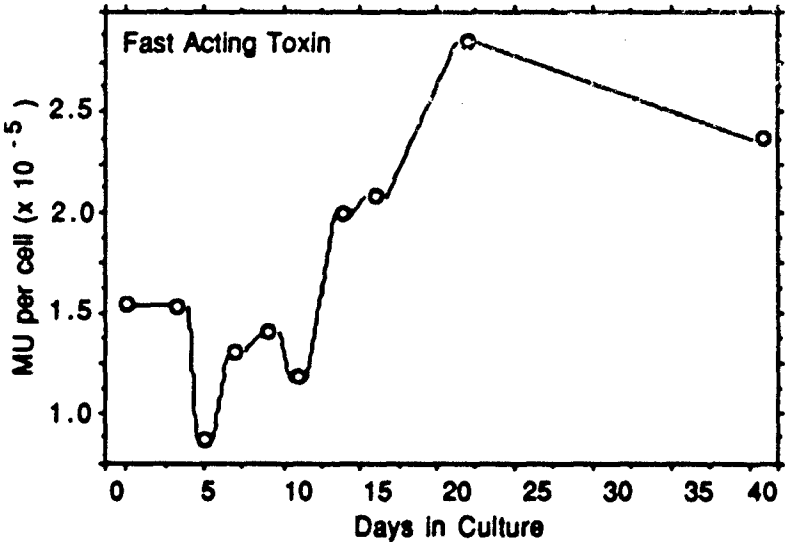


Figure 11. Cell potencies of Fast Acting Toxin as determined by mouse bioassay. Total MU of Fast Acting Toxin per cell, inset gives correlation between MU FAT/cell and total lipid content per cell.

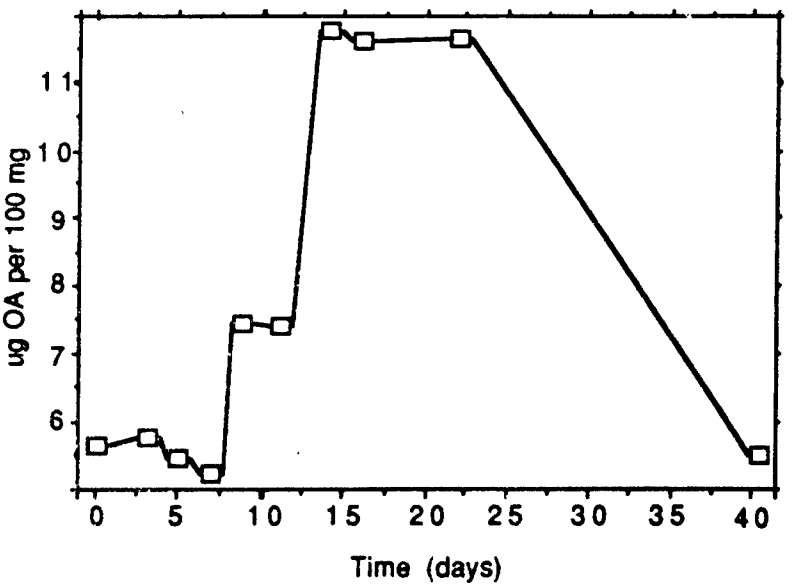


Figure 12. Cellular content of okadiac acid as determined by enzyme linked immunosorbent assay (ELISA) as quantity of extractable OA per 100 mg dry cells.

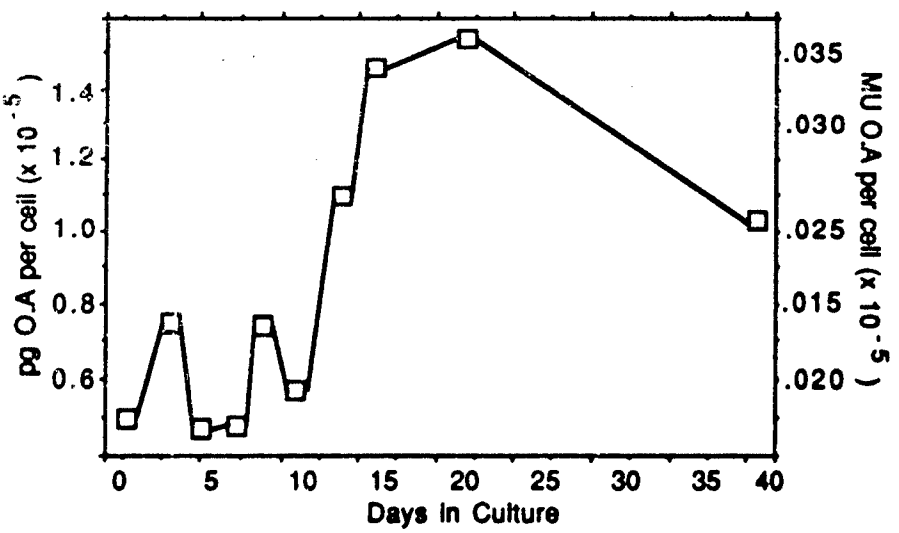


Figure 13. Cellular content of okadiac acid as determined by enzyme linked immunosorbent assay (ELISA) amount of OA per cell and MU OA per cell, this estimation is based on previously reported LD50's of 4 µg/MU. The inset gives the correlation coefficient for pg OA/cell vs total pg lipid/cell.

It is interesting to note the similarities in the ratios of water soluble toxin to lipid-soluble toxin between the two toxic dinoflagellates *P.*

concavum (SIU 882a) and *G. toxicus* (SIU 175). The ratio of FAT:OA for *P. concavum* 882a from this study was 74:1, which is not unlike ratios of maitotoxin:ciguatoxin in cultured *G. toxicus* 175. Another important point to note is the inverse relationship between toxin production and the uptake of phosphate (r = -0.96). This suggests that the phosphate assimilation mechanism may cause a negative-feedback on *de novo* toxin synthesis. However, low toxicity may be a result of increased rates of division in early log-phase which is the result of the same amount of toxin, being distributed amongst more cells giving the illusion of decreased toxicity (Anderson *et al.*, 1990). If this were the case one would expect to observe a rise and fall of toxin levels throughout the log-phase. So, this hypothesis should be tested using synchronous cultures.

Conclusions. The changes in physiology over the complete growth cycle of *P. concavum* (SIU clone 882a) were significant and consistent between each measured parameter. Curves for cellular content of protein, carbohydrates, lipids, pigments and toxins were very comparable. Nevertheless, toxin content per cell and total lipid content per cell displayed the highest correlation. These results suggest maximal cellular fitness and toxicity occur at mid-log phase growth. During this phase of the growth cycle the cells were shown to contain the highest cellular content of protein, pigments and toxin. These results also reveal the overwhelming production of water-soluble toxins vs lipid-soluble toxins (74:1) per cell as grown in culture. This relationship appears similar to the cellular content of maitotoxin:ciguatoxin in cultured *G. toxicus*. Also, the findings of this study confirm the report of OA in *P. concavum* cells by Dickey,[62] and further supports the conclusion that *P. concavum* may contributes to the disease ciguatera.

Fractionation, Separation and Assay of Components from *Gambierdiscus toxicus* (Clone 175)

During 1990, we initiated a series of liquid-liquid separations of the major toxic fractions of *G. toxicus* (175) in order to accumulate quantities sufficient for completion of purification. Representative samples of crude methanol extract and the water soluble and ether

soluble toxic fractions were delivered to USAMRIID for assay using rats (Table 2.).

Table 2.
Crude *Gambierdiscus toxic* fractions delivered to USAMRIID for rat assay (Harvest 3B89D 5(AB).

TOXIC FRACTIONS (in MeOH)	VOL. (ml)	TOTAL WT(mg)	MU (∝g)	TOTAL MU's	MU/µl
1 (5AB) Crude MeOH Extract	2.9	2.15	2.23	964	0.332
2 (5B) Water Sol. (Crude MTX)	1.0	0.34	0.30	1133	1.133
3 (5B) Ether Sol. (Crude CTX/MTX)	1.0	0.71	0.806	881	0.881

In processing these separations, some of which are listed in Table 3, we noted that there was some variation in number of mouse units per mg cells harvested (crude MeOH extracts, WST, and EST). These variations appear to have been due to differences in the physiological status of cells at the time of harvest rather than alterations of the extraction and separation procedures. The results from seven separate extractions of harvest 3B89D (1AB-7AB) show the good consistency obtained with our procedure.

A total of 1772.8 mg (2,928,995 MU's) of semipurified toxins have been accumulated. This amount represents 8.79 mg of pure MTX (equivalents). We anticipate that when the next series of purification steps are completed on all toxic fractions, we will have about 70 mg of toxin with an equivalent potency of about 2,300,000 MU's (estimated MU = 0.03 mg or 10% pure). Several WST fractions have been purified to a level of 6% purity (MTX equivalent).

Table 3.
Results from liquid-liquid separations and mouse assays completed during extraction of toxins from *G. toxicus* 175. WST= water soluble toxin (crude MTX); EST= ether soluble toxin (crude CTX+MTX); MU= mouse unit (one MU= LD50/20g mouse, ip injection)

HARVEST	CELLS	MEOH EXT.		WST		EST	
	(mg)	(mg)	(MU)	(mg)	(MU)	(mg)	(MU)
5A88D1AB	2000	537	111991	63.8	45425	20.0	3641
5A88D2AB	2000	522	101754	68.0	38558	14.0	2064
11A88D2AB	2000	690	298314	39.0	83459	65.0	49815
3B89D1AB	2000	645	274936	33.8	125000	62.0	94804
3B89D2AB	2000	667	263116	35.7	124468	72.0	99042
3B89D3AB	2000	669	232292	33.1	104564	57.0	98038
3B89D4AB	2000	653	292600	29.9	104444	69.0	83334
3B89D5AB	2000	648	292583	32.0	107172	69.0	86812
3B89D6AB	2000	666	288312	35.0	128621	77.5	66601
3B89D7AB	2000	642	277922	35.0	116723	76.5	90823
4A89D1AB	2000	603	154615	27.0	105556	57.5	69445
5B89D1AB	2000	591	115205	24.0	65615	86.0	61209
6A89D1AB	2000	594	118021	31.0	48613	58.0	44753
6C89D1AB	2000	666	271837	37.5	94444	44.5	69694
7A89D1AB	2000	663	162580	26.0	60496	47.5	50174
8A89D1AB	2000	591	144287	40.5	92857	60.0	44444
9A89D1AB	2000	681	207306	39.0	166981	52.0	69418
11B89D1AB	2000	744	154846	30.0	42231	51.0	51052
1A90D1AB	2000	672	254545	32.0	132129	42.0	55642

TOTAL MU (WST + EST) = 2, 978, 161 or 74.1% No significant mouse units were found in other products of liquid-liquid separation.

We are currently selecting (clone 175) harvests for extraction which represent cells subjected to slight variations in growth conditions in hopes of determining the basis of variations in potency of the three fractions. Also, we have initiated a large-scale growth experiment using clone 175 to determine the time of maximum potency of each type of toxin during a complete growth cycle. Such an experiment has been completed using *G. toxicus* (clone 350).

Much as we previously reported for separation of clone 350 we experience a loss of toxicity (ca. 25%) as we progress from crude extracts to the two toxic endproducts. We have no proven explanation for this loss; however, we speculate that the loss may be due to: alteration of the toxic compounds, loss of toxins in the rather heavy "non-toxic" end products (H_2O, hexane and ESAF), and/or there is a

positive synergistic effect of two or more toxins present in the crude methanol extract.

Fractionation, Separation and Assay of Components from *Gambierdiscus toxicus* (Clone 350)

A new chlorophyll–a from clone 350. In the process of extracting toxin from the 350 clone of dinoflagellates we discovered that there was an additional type of chlorophyll-a present which was different from the normal type. Preliminary ^{1}H NMR data indicated that the new type of chlorophyll differed in having an extra ring structure and information on this pigment was presented at the Dinoflagellate Conference in Sweden.[63]

Because of its uniqueness, we looked further at the structure and presence of the new pigment. Chlorophyll was extracted from several mass cultures and purified first by hexane extraction. The hexane extract was dried down and taken up in methanol. The methanol solution was applied to the preparative HPLC and monitored at 270 nm. For elution from the column a complex gradient starting with 90% acetonitrile-10% ethyl acetate. This changed to 100% ethyl acetate in 10 minutes, after which the ethyl acetate was replaced by methanol in the next ten minutes. Six distinct peaks appeared in the chromatogram at 9.5, 18.0, 19.5, 21, 22.0, and 26.5 min. The largest at 19.5 minutes proved to be the blue pigment. A second "clean-up" run of that peak using the same gradient conditions produced a peak of 30 mg of blue pigment. This was subjected to both ^{1}H and 13C NMR.

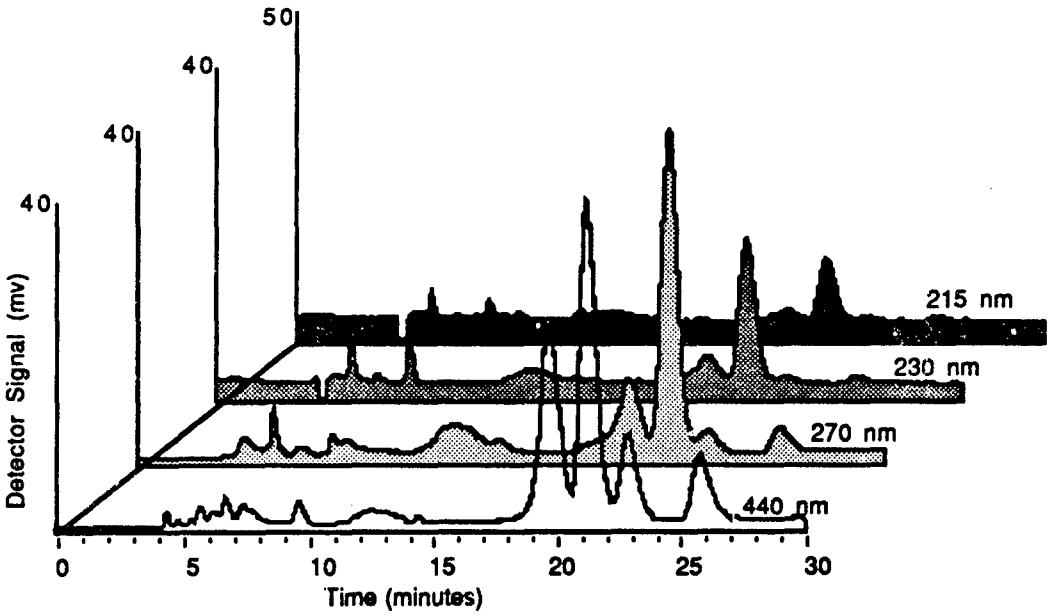


Figure 14. Analytical HPLC chromatograms of hexane fraction of *Gambierdiscus toxicus* (GT-350) run at 0.3 ml/min on Radial Pak μBONDASPHERE 15μ C18 300Å column, using a Waters 490 programmable detector set at 215, 230, 270 and 440 nm. Three chlorophyll peaks occur at 19.5, 21.5 and 22.8 min. The peak at 26.1 is β-carotene.

On the analytical HPLC the chlorophyll peaks appeared as three distinct peaks The Chl(s)-a eluted as three distinct peaks, #C-1, #C-2, and #C-3 at 19-26 minutes (Figure 14 and Table 4).

Table 4
Elution Times of Chlorophylls-a from Dinoflagellate Extracts on Analytical, Semi-preparative and Preparative HPLC

	Peak #C-1	Peak #C-2	Peak #C-3
Analytical	19.5	21.5	22.8
Semipreparative	28.5	29.1	30.1
Preparative	26.0	29.0	33.0
Color of Peak	Pea green	Blue green	Kelly green

Milligram amounts of the pigments were purified by peak selection and rechromatography. Interestingly enough, the initial peaks separated on the HPLC contained lipid plus pigment. The relative colors were as shown in Table 3. The lipids are easily removed from the

pigment by re-chromatographing, using a mixture of acetonitrile/ethyl acetate/methanol (1:1:1) isocratically and selecting peaks (see Figure 15). However, experience has shown that the pigment is more stable and stores better without allomerization when the lipids are present.

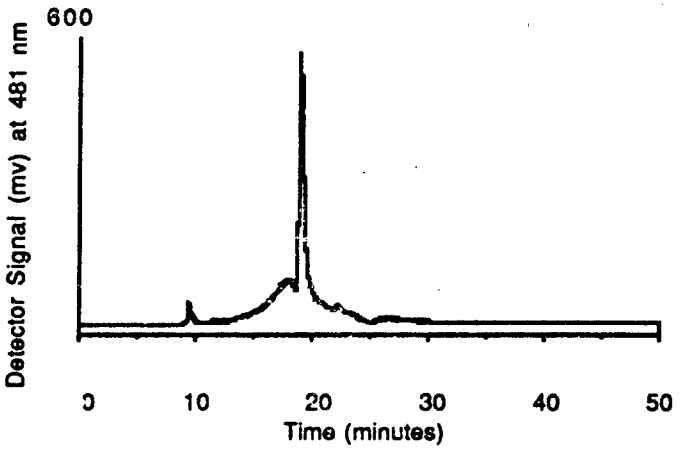


Figure 15. HPLC separation of purified chla$_{666}$ by selecting single peak at 19.5 minutes on preparative system using acetonitrile/ethyl acetate/methanol (1:1:1) in an isocratic mode. The large peak at 19.5 is chlorophyll-a$_{666}$.

Upon further purification and elimination of some of the lipids, it was found that the peaks contained varying amounts of two different chlorophylls-a, one appearing deep grass-green having a major absorption peak at 673 and another, appearing brilliant cobalt-blue, having a major absorption peak at 666. Hence to discriminate the two we have termed them simply chlorophyll-a$_{666}$ (chla$_{666}$) and chlorophyll-a$_{673}$ (chla$_{673}$). After purification, the two chlorophylls were dried, then taken up in deuterated methanol and examined on 500 MHz for ^{1}H (Table 5) and on the 300 MHz for ^{13}C (Table 6).

Table 5
^1H NMR Resonances of Green (673) and Blue (666) chlorophyll isolates from *Gambierdiscus toxicus* (GT-350)

Proton	Chlorophyll-a_{673}	Chlorophyll-a_{666}
β	9.60s	9.60s
α	9.46s	9.38s
δ	8.61s	8.38s
2	7.98dd	7.97dd
2'	6.29d	6.18d
2"	6.18d	5.99d
10	6.25-5.5s	-
P-2	5.2t	5.0t
8	4.46q	4.32q
P-1a	4.51t	4.12t
P-1b	4.45m	4.08m
7	4.15dd	4.55dd
10 OMe	3.70s	3.68s
4 CH2	3.70q	3.74q
5Me	3.60s	3.58s
1Me	3.40s	3.31s
3Me	3.22s	3.28s
7a	2.6, 2.45m	2.26t
7b	2.25, 2.22m	2.26t
8Me	1.78d	1.43d
4Me	1.68t	1.65t
P-3Me	1.58s	1.48s
Phytyl Me's	0.84, 0.78	0.84, 0.78

Integration of the ^1H peaks of NMR indicates that fraction C-1 consisted of 38 mg of a linoleic acid derivative and 14.5 mg of green chlorophyll-a_{673} as described in the literature. Peak C-2 consisted of a linoleic acid derivative and blue chlorophyll-a_{666} and peak C-3 consisted of lipid and both chlorophyll-a_{673} and chlorophyll-a_{666}

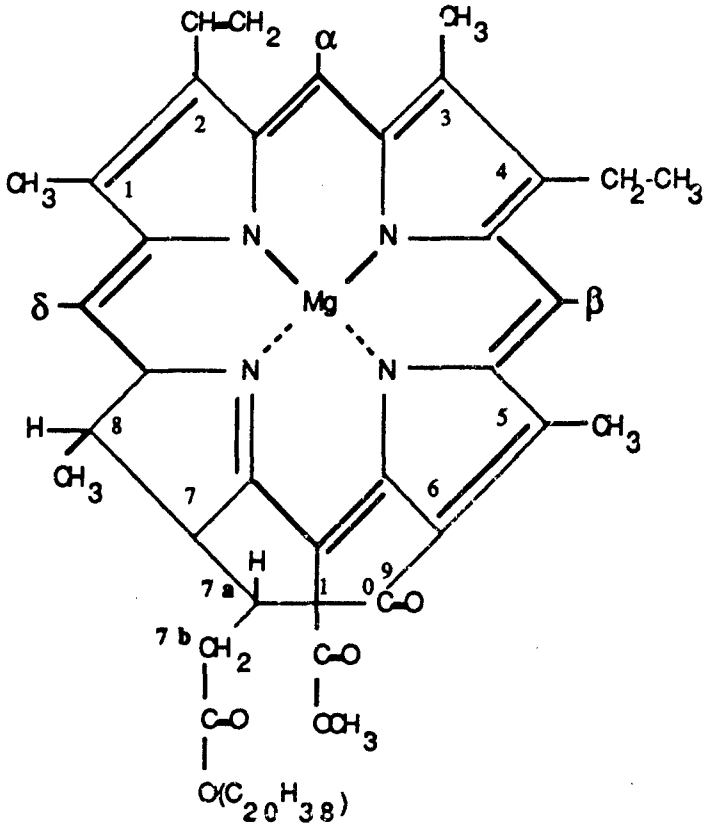


Figure 16. Diagram of structure of new chlorophyll isolated from dinoflagellates.

Upon inspection of the proton data it was observed that the blue pigment did indeed contain the porphyrin ring structure. The ring low field methine signals, the 2 vinyl group and ring methyl groups all match very closely with traces of standard chlorophyll a_{673}. Furthermore, all the distinguishable resonances from the phytyl chain are in place. However several differences can be seen; (1) the 6.25s is completely missing, (2) the 2.6 and 2.45 methylene signals are missing and (3) several resonances in ring IV (H-7, H-8, 8-Me) are significantly shifted. Furthermore in the blue chlorophyll trace, a large resonance at 2.26 is coupled to the 4.55 ppm signal, something not observed in standard chlorophyll a. With these data we affirm the following structure (Figure 16) for the blue chlorophyll a_{666}, which amounts to the formation of a new ring with covalent bonding of C-7a with C-10.

To obtain a ^{13}C spectrum of the blue chlorophyll we found it necessary to use a sample of about 30 mg. This gave significant differences between the resonance peaks of the pigment and those of the accompanying lipids. The lipid peaks were predetermined from samples of almost pure lipid, extracted from the pigments. The following is a listing of the peaks of the proton-decoupled ^{13}C spectrum of the chlorophyll a_{666} in $CDCl_3$ which were clearly resolved in about 12,000 transients, nearly three hours of accumulation.

Table 6
^{13}C Assignments for Standard Chlorophyll-a_{673} and the New Blue Chlorophyll-a_{666}

	Standard Green Chlorophyll a_{673}	Blue Pigment Chlorophyll a_{666}
P-3	143.0	143.0
C-2a	130.4	130.24
C-2b	119.9	120.21
P-2	117.7	117.6
C-β	107.8	107.49
C-α	100.1	102.16
C-δ	92.6	93.86
C-10	65.3	64.95
P-1	61.5	61.57
C-10b	53.0	52.93
C-7	50.4	52.31
C-8	49.7	48.41
P-4	39.8	39.73
P-14	39.4	39.36
P-10, P-8, P-12	37.4	37.41, 37.36, 37.27
P-6	36.6	36.66
P-11	32.8	32.78
P-7	32.7	32.66
C-7a	30.9	45.18
C-7b	28.0	31.43
P-15	28.0	27.96
P-5	25.0	25.0
P-13	24.8	24.79
P-9	24.4	24.44
C-8a	23.6	22.46
P-15a, P-16	22.7	22.70, 22.60
C-4a, P-7a, P-11a	19,7	19.72, 19.62, 19.49
C-46	17.5	17.61
P-3a	16.2	16.01
C-1a, C-5a	12.6, 12.3	12.38
C-3a	11.0	11.15

In Table 6 the ^{13}C resonances obtained for the chlorophyll a_{666} are shown along with those for the standard green chlorophyll a_{673} and clearly show certain peaks which undergo a significant shift.

Table 7
Relative Concentrations of Chlorophylls-a HPLC Peaks from Extracts of a Dinoflagellate *Gambierdiscus toxicus* (GT-350)

	Peak #C-1	Peak #C-2	Peak #C-3
Color Crude Peak	Pea green	Blue green	Kelly green
Amt of Chl_{666}	0.0 mg	10.5 mg	2.1 mg
Amt of Chl_{673}	14.5 mg	0.0 mg	5.0 mg
Amt of Lipid	38.0 mg	7.3 mg	33.9 mg

The mass cultures examined and the content of chlorophylls-a per unit weight are detailed in Table 7.

Table 8
Specific Concentration of Chlorophylls-a in the Four Clones of Dinoflagellates Examined

SPECIES (SIU CLONE NO.)	BLUE	GREEN
	mg Chl_{666} /gm	mg Chl_{673} /gm
G. toxicus (350)	0.29	0.40
G. toxicus (175)	19.51	0.01
P. concavum (364)	6.30	1.35
O. lenticularis (874)	0.51	0.01

Discussion and conclusions. This investigation has detailed a new procedure for the quantitative separation of chlorophyll(s)-a from dinoflagellates which can be used on other organisms equally well. The structural characterization of a new type of chlorophyll-a is another outcome of this study that is of considerable import. Our research has shown that is present in several clones of dinoflagellates. In fact, dried cells with larger content of blue chlorophyll pigment were much darker in color than those with less. It would be of interest to know if it is present outside of the dinoflagellates or if like peridinin this is pigment which is more or less specific to this group.

From the small number of clones that we have examined so far, we surmise that both types of chlorophyll are produced but the concentration may vary depending upon the growth conditions. This aspect needs further examination. Even more intriguing is the role of

this new kind of chlorophyll-a in the photosynthetic system in the dinoflagellate.

Separation of GT350 Toxic Material-GT-1.

Recently we have begun work on isolating and purifying the CTX from GT-350. We have found that this toxin can be extracted with hexane and in a relatively light-weight fraction (≈0.1%). Further purification of the material on C-18 columns and subsequent HPLC reveals that the major contaminant is C-18 lipid, a major component of the 350 cell system. Attempts to further purify this fraction on C-18 continue.

Separation of GT350 Toxic Material-GT-4.

A sample consisting of 200,000 MU from GT-350 was processed pas the 1 μg/MU level. Repetitions of the same step were utilized to help purify the sample. Toxicity tests indicated that mouse units were lost after each step of the purification process and even upon storage of th precipitate. Thus even though we lost most of the toxicity, we are still confident that we have the toxic moiety (albeit modified) in the purifie sample. After the final purification step the sample weighed 1.4 mg. A proton spectrum was easily achieved in five minutes using the VXR-50 spectrometer. Running the same sample for 66 hours in the VXR-300 instrument produced a ^{13}C spectrum with 135 lines.

The proton spectrum showed 10 protons in the olefinic region, 5.0 t 6.0. Although protons on carbons bonded to a keto carbon also adsorb in this range, the couplings as provided by a 2 ` COSY spectrum does distinguish between the two. The proton res ıances and their coupled resonances indicate the following:

Table 9
Table of Projected Groups from Specific ^{1}H NMR Peaks

δ	COUPLED TO	TYPE OF STRUCTURE	DIAGRAM
5.08	-	exomethylene	T
5.20	-	exomethylene	T
5.12	3.48, 1.6	trisubstituted olefinic bond	U
5.12	2.05	trisubstituted olefinic bond	V
5.25	4.15	proton on keto carbon bounded to carbon bearing oxygen on 6 membered hydroxylated ring	W
5.29, 5.30, 5.30, 5.30	2.78, 2.0, 1.97	chain segment of two disubstituted olefinic bonds	X
5.45	3.40	proton on keto carbon bounded to carbon bearing oxygen on 6 membered non-hydroxylated ring	Y

Table 10
Table of Projected Groups from Specific ^{13}C NMR Peaks

δ range	No. Carbons	Structure Type
24.96-9.76	25	Methyl
41.13-25.30	30	Methylene & methines
59.47-42.02	13	Carbons bonded to N
84.33-60.90	23	Carbons bonded to O
104.41-96.76	6	Keto carbons (possibly some high field C=C such as exomethylenes
148.1-107.18	24	Double bonded carbons
170.79-151.50	13	Carbonyls and carboxylic carbons

The data from the ^{13}C accumulated spectra revealed 55 carbons in the aliphatic range, forty two carbons sp3 bonded to oxygen and nitrogen and 37 double bonded carbons. This distribution is quite different from any data published to date, especially in the double bond range. The MTX spectra published by Yasumoto only shows 8 double

bonded carbons. The range in which the 134 signals appear is given along with the general structure types.

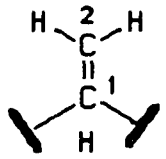
In a limited time we assigned a terminal chromophore, $C_{11}H_{14}N_3O_3$, close replica of calimycin, the calcium ionophore as the other terminal end; and fragments containing 5 isolated olefinic bonds, $C_{17}H_{24}O$ (total) The molecular weights and carbon types within these 3 groups are shown in the following table.

Table 11
MTX from GT-350 Structure Groups Assigned from ^1H and ^{13}C Spectra

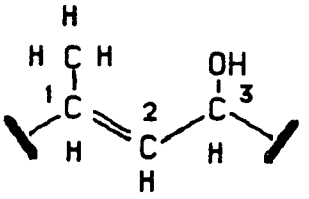
Group	Molecular F	MW	C=C	CCO	CO	CN	CH_2,CH	CH_3
Terminal chromophore	$C_{11}H_{14}N_3O_3$	236	9	0	1	0	1	0
Calcium ionophore	$C_{30}H_{3}$[illegible]$_4O_5$	533	14	1	2	1	8	5
Olefinic fragments	$C_{17}H_{24}O$	244	10	0	1	0	4	2
Total Assig. to date		1013	33	1	4	1	13	7

The assigned groups constitute about 1,000 MW leaving 1,800-2,00 MW in the central chain section of the molecule. With only 4 low field carbons yet to be assigned, it does not appear that any more conjugate ring structures exist. The presence of proton resonances in the 3.4-2.8 ppm range also suggest a bridged ring structure as seen in atropine or quinine containing 1 or more nitrogen atoms. Ten additional carbon resonances in the 42-59 ppm region are to be assigned, which probabl are some carbons bearing nitrogen. We further assume probably 40-5 oxygen atoms on carbons and hydroxal groups, as 22-25 carbons in th 60-85 ppm regio: are yet to be assigned. Analysis of the ^{1}H2D COSY spectrum is invaluable in this effort.

Partial structures were assimilated from ^1H 1-D and ^1H-2D COSY spectra. Carbon assignments are made using lines from the 66 Hz accumulated ^{13}C spectra and ^{13}C data on standard samples which we have acquired on the same instruments.



FRAGMENT T



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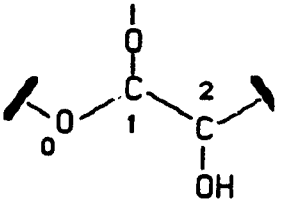

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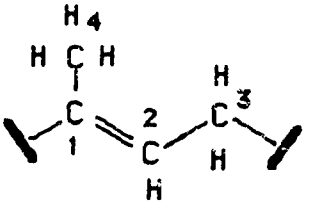

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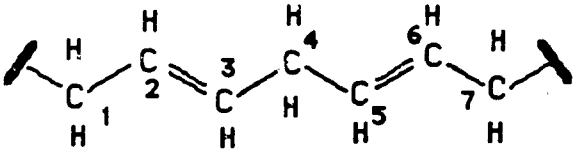
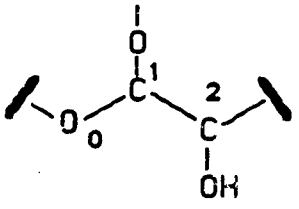


FRAGMENT X



FRAGMENT Y

Figure 17. Fragments T through Y determined from ^{1}H NMR spectrum Projected from ^{1}H, ^{13}C, and ^{1}H COSY Spectrum.

Table 11
Tentative Assignments of ^{13}C and ^{1}H Shifts for Fragments T through Y.

	Carbon δ	Proton δ	Proton δ	Code	
1	115.08			1T	-C- (=C)
2	107.18	5.08	5.20	2T	=C (H2)
3	107.68	1.6	1.6,1.6	1U	-C-H3
4	125.45	5.12		2U	-C= (H)
5	73.47	3.48		3U	-C- (H, 0H)
5	12.56	1.6	1.6,1.6	4U	-C-H3
6	108.66	1.7		1V	-C= (H)
7	123.96	5.12		2V	-C= (H)
8	28.14	2.02	2.05	3V	-C- (2)
9	12.31	1.6	1.6	4V	-C(H3)
9				0W	-O-C
10	103.03	5.25		1W	-C- (H, -O-)
11	82.24	4.15	OH	2W	-C- (H, OH)
12	26.62	2.02	2.0	1X	-C- (H2)
13	129.48	5.30		2X	-C= (H)

14	129.84	5.29		3X	-C= (H)
15	25.30	2.75	2.75	4X	-C- (H2)
16	130.51	5.30		5X	-C= (H)
17	131.07	5.30		6X	-C= (H)
18	26.00			7X	-C- (H2)
19				0Y	-O-C
20	98.23	5.45		1Y	-C- (H, -O-)
21	64.64	3.40		2Y	-C- (H, OH)

Using both the ^{13}C, ^{1}H, and 2D-COSY spectra a preliminary assignment can be made for one of the terminal ends of the molecule.

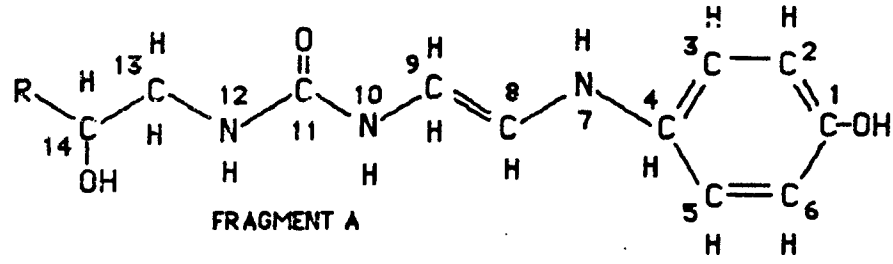


Figure 18. Projected Structure of Terminal Fragment A Determined from ^{1}H, ^{13}C, and ^{1}H COSY Spectrum

Table 12
Tentative Assignments of ^{13}C and ^{1}H Shifts for Terminal Fragment A

	Carbon δ	Proton δ	Proton δ	Proton δ	
1	151.50		0H	1A	=C- (OH)
2	128.16	7.61		2A	=C- (H)
3	125.32	7.71		3A	=C- (H)
4	148.1			4A	-C= (H)
5	125.32	7.71		5A	=C- (H)
6	128.16	7.61		6A	=C- (H) (H)
7					-N- (H)
8	147.9	7.40		7A	-C= (H)
9	153.78	7.95		8A	-C= (H)
10					-N- (H)
11	170.79			9A	-C- (=O)
13	44.79	4.26	4.26	10A	-C- (H2)
12					-N- (H)
14	67.26	3.85	OH	11A	-C- (H, OH)

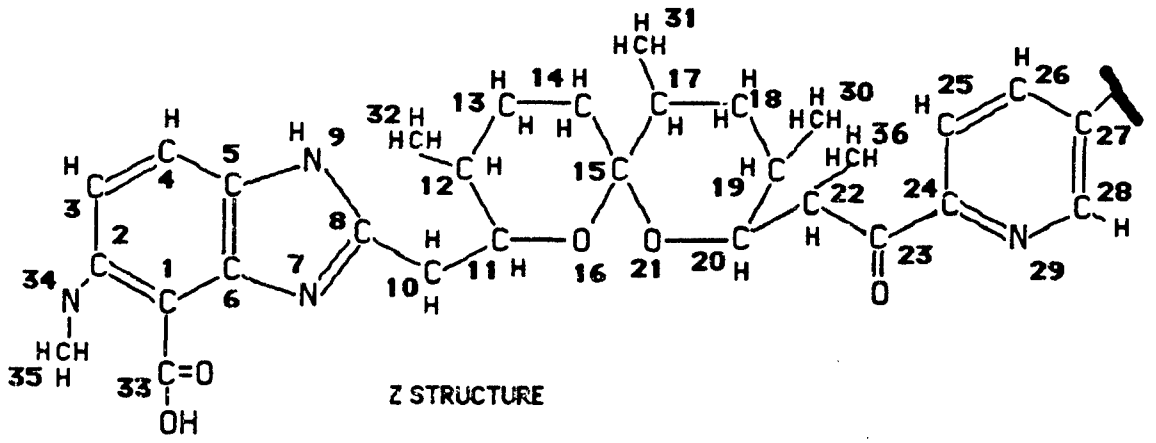


Figure 19. Projected Structure of Terminal Fragment Z Determined from ^{1}H, ^{13}C, and ^{1}H COSY Spectrum

Table 13
Tentative Assignments of ^{13}C and ^{1}H Shifts for Terminal Fragment Z

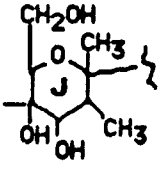
No.	Carbon δ	Proton δ	Proton δ	Proton δ
1	96.76			
2	126.96			
3	122.46	7.37		
4	142.1	7.45		
5	137.2			
6	139.24			
7				
8	160.8			
9		8.55		
10	32.45	2.48		
11	66.95	4.26		
12	29.46	1.25		
13				
14				
15	104.41			
16				
17				
18				
19		1.70		
20	64.40	4.30		
21				
22	34.88	2.48		
23	170.1			
24	142.1			
25	109.84	6.84		
26	103.41	7.22		
27	99.71			
28	133.39	7.73		
29				

30	10.82	0.85
31	11.39	
32	14.01	0.80
33	161.52	
34		
35	42.02	
36	16.56	

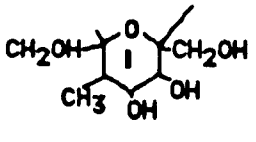
In this section we give the structural determination of another part of the isolated molecule from *G. toxicus* 350 utilizing our NMR data consisting of the ^{1}H proton spectrum, the 66 hr accumulated ^{13}C spectrum and the COSY 2D ^{1}H spectrum. Again, we base our proposed structure on comparison of NMR data we obtained on our instruments with model compounds such as the antibiotics, monensin, sodium ionophore X-537A, calcium ionophore A-23187, novobiocin, etc; the saccharides such as galactose, trehalose, xylose and nitrogen containing receptor site deactivators such as reserpine, bepridil, diltiazem and others.

Immediately apparent in the ^{13}C spectrum were six signals in the 105-96 ppm range indicating keto carbons of six-membered ether rings such as found in trehalose and some antibiotics.

Table 14
Structure and Assignments for part J and I



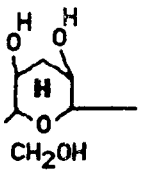
Fragment	1H	13C
J1	-	Q
J2	3.80	73.[illegible]
J3	4.21	71.54
J4	1.38	34.49
J4Me	0.89	18.0
J5	--	Q
J6	1.41	19.3
J7	3.75 &3.81	62.14



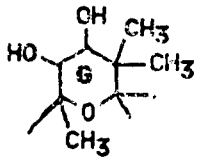
Fragment	1H	13C
I1	-	Q
I2	4.30	62.27
I3	4.15	62.14
I4	1.61	34.67
I4Me	0.99	09.76
I5	-	Q
I6	3.65, 3.75	62.14
I7	3.67, 3.72	62.14

Also approximately 30 signals between 60 & 85 ppm indicate the same. The COSY 2D ^{1}H spectrum further indicated that most of the proton signals in the 3.4-5.4 ppm were coupled to each other such as in the saccharides with a minimum of coupling to high field methylene and methine carbons. Some coupling to signals in the 1.6-1.3 ppm range indicated a limited number of CH and CH2's in the ether rings.

Table 15
Structure and Assignments for part H and G



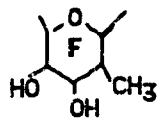
Fragment	1H	13C
H1	3.64	63.33
H2	3.83	69.10
H3	4.06	70.74
H4	4.0	64.64
H5	-	Q
H6	3.74, 3.77	62.14



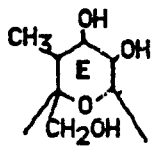
Fragment	1H	13C
G1	3.51	71.54
G2	-	34.88
G2Me	1.40, 1.39	10.31, 10.47
G3	4.21	67.26
G4	4.28	64.40
G5	-	Q
G6	1.38	16.5[illegible]

The formulation of 9 such ether rings utilize all the carbons in the ^{13}C spectrum between 60 and 85 ppm while it appears that some quarternary carbons in this range did not resolve. This is not uncommon and may be related to the limited solubility of the molecule. All of the protons arranged in the structure are indicated by the COSY 2D spectrum. Using the available data we have incorporated these ring structures B through J into a complex ring structure which we believe connects to the terminal end A.

Table 16
Structure and Assignments for part F and E



Fragment	1H	13C
F1	5.38	96.76
F2	3.40	72.45
F3	4.70	69.10
F4	1.37	35.07
F4Me	0.88	10.68
F5	4.10	84.12



Fragment	1H	13C
E1	4.27	69.32
E2	3.55	69.10
E3	3.90	66.71
E4	1.30	35.63
E4Me	0.85	14.01
E5	-	Q
E6	3.79, 3.84	62.14

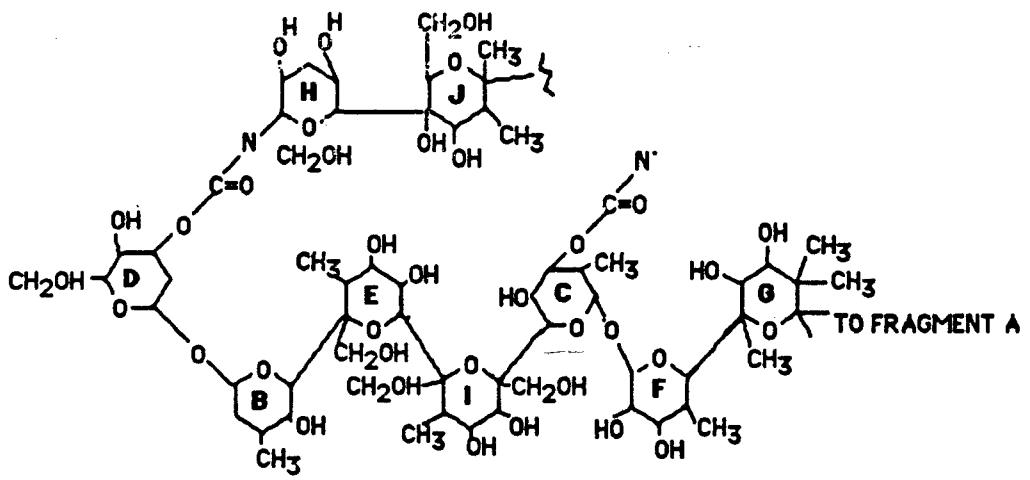


Figure 20. Coupling of Fragment A to Fragment B-J through ring G.

We recognize that coupling these structures together in a 1500 M fragment is not totally specific, however the structure we have indicated does satisfy all of the COSY 2D data. If short chain segme coupled the ether rings, there would be more ccupling with high fie signals. The COSY 2D did not indicate this. In this structure, 7 of th adjoining sites are made at a quarternary carbon, 2 through an oxy and ring G is coupled to fragment A.

Delivery of Toxins:

The following were delivered to USAMRIID prior to this report:

Table 17
Delivery of toxins to USAMRIID during 1990:

Lot No-Kind	Contract Item No		Amt	MU	Date
Lot GT175-Crude	Item 0001BS-1	0.458 g		1Jan90	
Lot GT175-Crude	Item 0001BS-2	0.458 g		1Jan90	
Lot GT175-Crude	Item 0001BS-3	0.458 g		1Jan90	
Lot GT175-Crude	Item 0001BS-4	0.458 g		1Jan90	
Lot GT350-Crude	Item 0001BT			30Jan90	
Lot GT350-GT-4	Item 0001BU			30Jan90	
Lot GT350-GT-1	Item 0001BV			30Jan90	
Lot 00-Aust RB	Item 0001BW	0.5 Kg		30Jan90	
Lot 00-Span Mack		Item 0001BX	1.0 Kg	27Feb90	
Lot GT175-Crude	Item 0001BY	2.15 mg		27Feb90	
Lot GT175-GT-4	Item 0001BZ	0.34 mg		27Feb90	
Lot GT175-GT-1	Item 0001CA	0.71 mg		27Feb90	
Lot GT175-Crude	Item 0001CB	1.27 g			
Lot GT175-Crude	Item 0001CC-1	0.8 g			
Lot GT175-Crude	Item 0001CC-2	0.8 g			
Lot GT175-Crude	Item 0001CC-3	0.8 g			
Lot GT175-Crude	Item 0001CC-4	0.8 g			

Travel Performed During this Period:

The Marine Toxins Session of the ACS Meeting (26-29 Aug 90) in Washington, D. C. was attended by Donald M. Miller, and subsequently Ft Detrick was visited (30-31 Aug 90) for the presentation of the Final Verbal Report for this contract.

According to pre-approved plans a field trip was conducted to collect dinoflagellates from the around the area of the coral sea. The first part of the trip was immensely successful. This success was due in part to the assistance of Dr. Michael Capra from the Queensland University of Technology (QUT). He arranged for the renting of vehicles from QUT, called ahead of time for access to transport out to collecting sites, arranged permits (copies attached as attachments a) for both Australia and Fiji and coordinated all aspects of the trip.

The schedule for the trip and collection from 14 sites was as follow

Date	Destination
13-19 Oct 90	Cairns Aust.
	•Collected Michaelmus Cay
	•Collected Green Island
20-22 Oct 90	Townsville Aust
	•Collected Kelso Reef
	•Collected Magnetic Island, Arthur Bay
	•Collected Magnetic Island, Picnic Bay
23-26 Oct 90	Mackay Aust
	•Collected Brampton Island
	•Collected Credlin Reef
27-30 Oct 90	Heron Island Marine Station
	•Collected Heron Island around entire island
3-6 Nov 90	Fraser Island
	•Collected Fraser Island
10-15 Nov 90	Vita Levu Island Fiji
	•Collected Vaileka Bay, Rakiraki, Fiji
	•Collected Saweni Beach, Lautoka, Fiji
	•Collected Tubakula Beach, Sigatoka, Fiji
	•Collected Koralevu Beach, Sigatoka, Fiji
	•Collected Suva Bay, Suva, Fiji.

We were very fortunate during the last part of the trip in that an outbreak of fish poisoning broke out in Fiji during the same time that we collected. As part of the trip a seminar was presented at The University of the South Pacific.

Work is now proceeding on the cultures brought back to our laboratory. At the present time 50 isolates of *Gambierdiscus toxicus* over 50 isolates of *Prorocentrum* sp. have been isolated. We are hopeful that most of these will survive to mass culture.

Also, another trip was made to the Florida Institute of Technology, Melbourne, Fl. for a load of sea water. As promised, they once again lowered their price per gallon at the pump for us to $0.69 per gallon. Altogether, we were able to load 1,444 gallons of water into the truck that we took for a total cost of $1,000. Our estimated travel costs we approximately $1,253.16 (which includes truck rental of $602.04; Fue

416.34; and meals and lodging at $234.78). The total amount spent in obtaining the 1,444 gallons of sea water was thus $2,253.16 and this averages out to a cost per gallon of $1.56. In a phone request for pricing on 29 October 1990 to Carolina Biological Supply we received a price of $21.82 per 5 gallon bucket, which averages out to $4.36 per gallon. Thus, our savings in getting this load of sea water was $4,053.

Visitations to the Laboratory

In May of 1990 the laboratory was visited by Dr. Richard Lewis from the Department of Primary Industries, Deception Bay, Australia. He was interested in observing our procedures and operations.

In July of 1990 the laboratory was visited by Dr. Christopher S. Lobban from the Marine Laboratory, University of Guam. Dr. Lobban, was seeking help on techniques since he has been awarded a Minority Grant from NIH to study *Gambierdiscus toxicus* around Guam.

Summary of Other Work:

Bob Adamson has completed his Master's degree thesis on "Extraction of Ciguatera type toxins from *Lutjanus bohar*, the Red Snapper".

Faiqa Hassan has completed her doctoral thesis on the effects of maitotoxin and ciguatoxin on primary culture cells of chick liver and brain.

Mark Jacyno has completed his masters paper on the effects of maitotoxin and ciguatoxin on frog nerve refractory period.

LITERATURE CITED

1. Lombet, A., Bidard, J.-N. and Lazdunski, M., (1987) Ciguatoxin and brevetox share a common receptor site on the neuronal voltage-dependent Na^+ channel, *FEBS Let.*, 219, 355-359.

2. Santostasi, G., Kuty, R.K. and Krishna, G., (1988) Loss of cell ATP due to increased uptake of calcium as a possible mechanism of maitotoxin-induc cell death in cardiozygotes, *FASEB J.*,

3. Palafox, N.A., Jain, L.G., Pinano, A.Z., Gulick, T.M., Williams, R.K. and Schatz I.J., (1988) Successful treatment of ciguatera fish poisoning with intravenous mannitol, *JAMA*, 259, 2740-2742.

4. Halstead, B.W., (1958) Fish poisonings - Their pharmacology, diagnosis, an treatment, *Clin. Pharmacol. Therap.*, 5, 615-627.

5. Halstead, B.W., (1967) Poisonous and Venomous Marine Animals of the Wor U. S. Government Printing Office, Washington, D. C, 1967.

6. Randall, J.E., (1958) A review of ciguatera, tropical fish poisoning, with a tentative explanation of its cause, *Bull. Mar. Sci. Gulf. Caribb*, 8, 236-267.

7. Lawrence, D.N., Enriguez, M.B., Lumish, R.M. and Maceo, A., (1980) Ciguate fish poisoning in Miami, *JAMA*, 244, 254-258.

8. Bagnis, R.A., Kuberski, T. and Laugier, S., (1979) Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific, *Am. J. Trop. Med. Hyg.*, 28, 1067-1073.

9. Tackett, C.O., (1982) Clinical and Epidemiologic Features of Ciguatera in the Caribbean, *U. S. Department of Health and Human Services Pamphlet*, PHS CDC, Atlanta, Georgia, 1982.

10. Capra, M.F., Flowers, A.E. and Cameron, J., (1988) The effect of ciguatoxin o teleost nerves, *Proceedings, World Congress on Animal, Plant and Microb Toxins (9th)*

11. Capra, M.F., Cameron, J., Flowers, A.E., Coombe, I.F., Blanton, C.G. and Hahn, S.T., (1988) The effects of ciguatoxin on teleosts, in *Sixth International Co Reef Congress*, Choat, J.H., Barnes, D., Borowitzka, M.A., Coll, J.C., Davies, P Flood, P., Hatcher, B.G., Hopley, D., Hutchings, P.A., Kinsey, D., Orme, G.R., Pichon, M., Sale, P.F., Sammarco, P., Wallace, C.C., Wilkinson, C., Wolanski, and Bellwood, O., Eds., 6th International Coral Reef Symposium Executive Committee, Townsville, Australia, 8-12 Aug 1989, 37-41.

12. Capra, M., Cameron, J., Flowers, A.E., Coombes, I.F., Blanton, C.G. and Hahn, S.T., (1988) The effects of ciguatoxin on mammalian nerves, *Proceedings, Sixth International Coral Reef Congress*, 4, 457-462.

13. Cameron, J. and Capra, M.F., (1987) Interim report on clinical and symptomatological studies performed on the victims of ciguatera poisoni in Sydney on 26th and 27th May 1987,

14. Cameron, J., Flowers, A.E. and Capra, M.F., (1988) The effects of ciguatoxin on nerve conduction parameters in humans and the laboratory rat, *Proceedings, Ninth World Congress on Animal, Plant and Microbial Toxins, Stillwater, OK.*

15. Sylva, D.P., de and Higman, J.B., (1980) A plan to reduce ciguatera in the tropical western Atlantic region, *Bull. Mar. Sci.*, 32, 139-153.

16. Baylet, R., Beccaria, C., Niaussat, P.M., Boyer, F. and Guillaud, M., (1978) Food poisoning by ciguatoxin in France, *Pathol. Biol.*, 26, 95-97.

17. Bagnis, R.A., (1968) Clinical aspects of ciguatera (fish poisoning) in French Polynesia, *Hawaii Med. J.*, 28, 25-28.

18. Li, K.-m., (1968) Action of ciguatera fish poison and multiple toxins in ciguatera fish species, in *Poisonous and Venomous Marine Animals*, Halstead, B., Eds., U. S. Government Printing Office, Washington, D. C.,

19. Banner, A.H., (1974) The biological origin and transmission of ciguatoxin, in *Marine Science. Bioactive Compounds from the Sea.*, Humm, H.J. and Lane, C.E., Eds., Marcel Decker, New York, 15-36.

20. Li, K.-m. and Au, A.T.C., (1968) Clinical diagnosis - Ciguatera poisoning, *Far East Med. J.*, 4, 313-315.

21. Hashimoto, Y. and Fusetani, N., (1968) A preliminary report on the toxicity of the amberjack *Seriola aureovittata*, *Bull. Jpn. Soc. Sci. Fish.*, 34, 618-625.

22. Yasumoto, T., Hashimoto, Y., Bagnis, R.A., Randall, J.E. and Banner, A.H., (1971) Toxicity of the surgeonfishes, *Bull. Jpn. Soc. Sci. Fish.*, 37, 724-734.

23. Yasumoto, T. and Kanno, K., (1976) Occurrence of toxins resembling ciguatoxin, scaritoxin, and maitotoxin in a turban shell, *Bull. Jpn. Soc. Sci. Fish.*, 42, 1399-1404.

24. Yasumoto, T., Nakajima, I., Chungue, E. and Bagnis, R.A., (1977) Toxins in the gut contents of parrotfish, *Bull. Jpn. Soc. Sci. Fish.*, 43, 69-74.

25. Bagnis, R.A., Loussan, E. and Thevenin, S., (1974) Les intoxications par poissons perroquets aux Isles Gambier, *Méd. Trop.*, 34, 523-527.

26. Yasumoto, T., (1990) New results on marine dinoflagellate toxins, in *Toxic Marine Phytoplankton Blooms*, Graneli, E., Sundstrom, B., Edler, L. and Anderson, D.M., Eds., Elsevier Press, New York,

27. Kodama, A.M., Hokama, Y., Yasumoto, T., Fukui, M., Manea, S.J. and Sutherland, N., (1989) Clinical and laboratory findings implicating palytoxin as cause of ciguatera poisoning due to *Decopterus macrosoma* (Mackeral), *Toxicon*, 27, 1051-1053.

28. Murata, M., Legrand, A.-M. and Yasumoto, T., (1989) A probable partial structure of ciguatoxin isolated from Moray eel *Gymnothorax javanicus*, *Tetrahedron Let.*, 30, 3793-3796.

29. Poli, M., (1985) Characterization of the *Ptychodiscus brevis* polyether binding component in excitable membranes, in *Toxic Dinoflagellates*, Anderson, D.M., White, A.W. and Baden, D.G., Eds., Elsevier North-Holland Press, New York, 357-362.

30. Yasumoto, T., Nakajima, I., Bagnis, R.A. and Adachi, R., (1977) Finding of a dinoflagellate as a likely culprit of ciguatera, *Bull. Jpn. Soc. Sci. Fish.*, 43, 1021-1026.

31. Adachi, R. and Fukuyo, Y., (1979) The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov collected in a ciguatera endemic area, *Bull. Jpn. Soc. Sci. Fish.*, 45, 67-71.

32. Yasumoto, T., Nakajima, I., Oshima, Y. and Bagnis, R.A., (1979) A new toxic dinoflagellate found in association with ciguatera, in *Toxic Dinoflagellate Blooms*, Taylor, D.L. and Seliger, H.H., Eds., Elsevier North-Holland, New York, 65-70.

33. Yasumoto, T., Oshima, Y., Murakami, Y., Nakajima, I., Bagnis, R.A. and Fukuyo, Y., (1980) Toxicity of benthic dinoflagellates found in coral reef, *Bull. Jpn. Soc. Sci. Fish.*, 46, 327-331.

34. Withers, N.W., (1981) Toxin production, nutrition, and distribution of *Gambierdiscus toxicus* (Hawaiian Strain), *Proceedings of the Fourth International Coral Reef Symposium*, 2, 449-451.

35. McFarren, E.F., Tanabe, H., Silva, F.J., Wilson, W.B., Campbell, J.E. and Lewis, K.H., (1965) The occurrence of a ciguatera-like poison in oyster, clams, and *Gynodinium breve* cultures, *Toxicon*, 3, 111-123.

36. Bergmann, J.S. and Alam, M., (1981) On the toxicity of the ciguatera producing dinoflagellate *Gambierdiscus toxicus* Adachi and Fukuyo isolated from the Florida Keys, *J. Environ. Sci. Health A1*, 6, 493-500.

37. Tindall, D.R., Dickey, R.W., Carlson, R.D. and Morey-Gaines, G., (1984) Ciguatoxigenic dinoflagellates from the Caribbean, in *Seafood Toxins*, Ragelis, E.P., Eds., American Chemical Society Symposium Series 262, Washington, D. C., 225-240.

38. Yentsch, C.M., Dale, B. and Hurst, J.W., (1978) Coexistence of toxic and nontoxic dinoflagellates resembling *Gonyaulax tamarensis* in New England coastal waters (NW Atlantic), *J. Phycol.*, 14, 330-332.

39. Schmidt, R.J. and Loeblich, A.R., (1979) Distribution of paralytic shellfish poison among Pyrrhophyta, *J. Mar. Biol. Assn. U. K.*, 59, 479-487.

40. Oshima, Y., Hayakawa, T., Hashimoto, H., Kotaki, Y. and Yasumoto, T., (1982) Classification of *Protogonyaulax tamarensis* from Northern Japan into three strains by toxin composition, *Bull. Jpn. Soc. Sci. Fish.*, 48, 851-854.

41. Bagnis, R.A., Chanteau, S., Chungue, E., Hurtel, J.-M., Yasumoto, T. and Inoue, A., (1980) Origins of ciguatera fish poisoning: a new dinoflagellate, *Gambierdiscus toxicus* Adachi and Fukuyo, definitively involved as a causal agent, *Toxicon*, 18, 199-208.

42. Dickey, R.W., Miller, D.M. and Tindall, D.R., (1984) Extraction of a water-soluble toxin from a dinoflagellate, *Gambierdiscus toxicus*, in *Seafood Toxins*, Ragelis, E.P., Eds., American Chemical Society Symposium Series 262, Washington, D. C., 257-269.

43. Carlson, R.D., Morey-Gaines, G., Tindall, D.R. and Dickey, R.W., (1984) Ecology of toxic dinoflagellates from the Caribbean Sea: Effect of macroalgal extracts on growth in culture, in *Seafood Toxins, Proceedings, American Chemical Society Conference on Toxins in Seafood*, Ragelis, R.P., Eds., 271-287.

44. Miller, D.M., Dickey, R.W. and Tindall, D.R., (1984) Lipid soluble toxins from a dinoflagellate, *Gambierdiscus toxicus*, in *Seafood Toxins*, Ragelis, R.P., Eds., American Chemical Society Symposium Series 262, Washington, D. C., 241-255.

45. Provosoli, L., (1968) Media and prospects for the cultivation of marine algae, in *Proceedings, Fourth International Seaweed Symposium*, DeVirville, D. and Feldman, J., Eds., Pergammon Press, Oxford, 9-17.

46. Steidiger, K.A., (1983) A re-evaluation of toxic dinoflagellate biology and ecology, in *Progress in Phycological Research*, Round, F.E. and Chapman, V.J., Eds., Elsevier Press, New York, 147-188.

47. Brand, L.W., Guillard, R.R.L. and Murphy, L.S., (1981) A method for the rapid and precise determination of acclimated phytoplankton reproduction rates, *J. Plankt. Res.*, 3, 192-201.

48. Bomber, J., (1990) Genetic variability in toxic dinoflagellates, in *Ciguatera Sea Food Toxins*, Miller, D.M., Eds., CRC Press Inc, Boca Raton, Fla, In Press.

49. Kochert, G., (1978) Quantitation of the macromolecular components of microalgae, in *Handbook of Phycological Methods: Physiological and Biochemical Methods*, Hellebust, J.A. and Cragie, J.S., Eds., Cambridge University Press, Cambridge, 189-195.

50. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F., (1956) Colorimetric method for determination of sugars and related substances, *Analyt. Chem.*, 28, 350-356.

51. Freeman, N.K., Lindren, F.T., Ng, Y.C. and Nichols, A.V., (1957) Infrared spectra of some lipoproteins and related lipids, *J. Biol. Chem.*, 203, 293-304.

52. Sperry, W.N. and Brand, F.C., (1955) The determination of total lipids in the blood serum, *J. Biol. Chem.*, 213, 69-76.

53. Jeffrey, S.W., Sielicki, M. and Haxo, F.T., (1975) Chloroplast pigment patterns in dinoflagellates, *J. Phycol.*, 11, 374-384.

54. Jensen, A., (1978) Chlorophylls and carotenoids, in *Handbook of Phycological Methods: Physiological and Biochemical Methods*, Hellebust, J.A. and Cragie, J.S., Eds., Cambridge University Press, Cambridge, 59-70.

55. Weil, C.S., (1952) Tables for convenient calculation of median effective dose (LD50 or ED50) and instructions for their use, *Biometrics*, 8, 249-63.

56. Strickland, J.D.H. and Parsons, T.R., (1972) A Practical Handbook of Seawater Analysis, *Fish Res. Bd. Canada Bull.*, 167, 310.

57. Bomber, J.W., (1987) Ecology, genetic variability and physiology fo the ciguatera-causing dinoflagellate *Gambierdiscus toxicus* Adachi and Fukuyo, PhD Dissertation, Florida Institute of Technology, Melbourne, FL, 1987.

58. Sokal, R.R. and Rohlf, F.J., (1981) Biometry, W. H. Freeman and Co., New York, NY, 1981.

59. Indelicato, S.R. and Watson, D.A., (1986) Identification of the photosynthetic pigments of the tropical benthic dinoflagellate *Gambierdiscus toxicus*, *Mar. Fish. Rev.*, 48, 44-47.

60. Miller, D.M., (1990) The ileum preparation, in *Ciguatera Seafood Toxins*, Miller, D.M., Eds., CRC Press, Boca Raton, FL, 110-115.

61. Anderson, H.J., Barrett, D. and Craver, R., (1949) *Arch. Intl. Pharmacol. Ther.*, Apr 1, 544-547.

62. Dickey, R.W., Bobzin, S.C., Faulkner, D.J., Bencsath, F.A. and Adrzejwski, D., (1990) The identification of okadiac acid from a Caribbean dinoflagellate, *Prorocentrum concavum*, *Toxicon*, 28, 371-377.

63. Miller, D.M., Tindall, D.R. and Venable, C.W., (1990) NMR spectroscopy of components isolated from *Gambierdiscus toxicus*, in *Toxic Marine Phytoplankton Blooms*, Graneli, E., Sundstrom, B., Edler, L. and Anderson, D.M., Eds., Elsevier Press, New York, 305-310.

GLOSSARY

ACN	Acetonitrile
ACN-insoluble	Acetonitrile insoluble fraction
ACN-soluble	Acetonitrile soluble fraction
ANOVA	Analysis of variance
COSY	Correlated Spectroscopy
CTX	Ciguatoxin
DEPT	Distortionless Enhancement Polarization Transfer Plot
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunoabosrbant assay
ESAF	Ether soluble acetone filtrate
ESAP	Ether soluble acetone precipitate
FAT	Fast Acting Toxin
GT-1	One of two toxins found in CTX group from *G. toxicus.*
GT-2	One of two toxins found in CTX group from *G. toxicus.*
GT-3	One of two toxins found in MTX group from *G. toxicus.*
GT-4	One of two toxins found in MTX group from *G. toxicus.*
HPLC	High Pressure Liquid Chromatography
IEU	Ileum Equivalent Units
OA	Okadiac Acid
MTX	Maitotoxin
MU	Mouse units
NMR	Nuclear Magnetic Resonance
P-	Abbreviation for Preparative
PPM	Parts per million
PSS	Physiological Saline Solution
SK	Acronym for Sep Pak separation of toxins
SP-	Abbreviation for Semipreparative
TEA	Tetraethylammonium
TLC	Thin layer chromatography
WSAF	Water soluble acetone filtrate
WSAP	Water soluble acetone precipitate
XTL	Acronym for crystalization step

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